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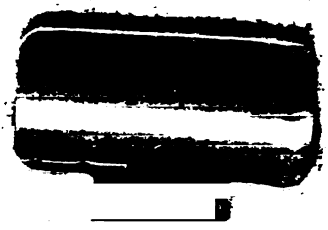

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Being reprints of studies published in 1912.

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AN EXPERIMENTAL STUDY OF RACIAL DEGENERATION IN MAMMALS TREATED WITH ALCOHOL*

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NEW YORK

It is recognized, by most observers who have studied the subject, that alcohol may play an important rôle in the causation of monstrosities and of structural defects predisposing to later disease. This view is based largely on observations on defective human beings, and the probability of its truth is sufficiently established to warrant further careful experimental analysis.

The quality of an offspring depends on two factors, the perfection of the germ cells from which it arises and the nature of the environment in which it develops. Diseased and weakened germ-cells give rise to a defective individual under all circumstances, while perfect germ-cells produce a perfect offspring *only* when the embryo develops in a normal or favorable environment. These facts may be readily demonstrated in lower vertebrates in which the development of the egg is outside the mother's body. The egg or spermatozoon in such cases may easily be chemically modified or injured before fertilization, and the embryo itself may be affected in various ways during its development by subjecting it to unusual surroundings, either physical or chemical. In other animals, such as mammals, in which the embryo develops internally, the proposition likewise holds true. In these animals, however, the problem is more difficult to completely analyze. The reactions of the parental body, the secondary conditions induced by the experimental treatment and other sources of error should be fully considered in determining whether an effect shown by the offspring is directly due to the applied stimulus or to secondary conditions. In the lower vertebrates it has been shown that given doses of certain substances induce definite developmental defects. The defects are directly due to the treatment. Is it possible by the addition of certain chemicals to the mammalian body to obtain similar definite changes in either the germ-cells or the developing embryo?

In the present paper I shall endeavor to show that alcohol does act directly on the germ-cells of mammals to a sufficient degree to render them incapable of producing normal offspring, and further, that similar treatment administered to the pregnant female may likewise act directly on the developing embryo so as to modify its resulting structure.

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*From the Anatomical Laboratory, Cornell University Medical College.
*Manuscript submitted for publication May 19, 1912.

First to appreciate fully the general status of the problem it is well to consider in a somewhat critical manner the literature pertaining to the actions of alcohol and other substances on the reproductive glands and developing embryos of man and lower animals.

DISCUSSION OF LITERATURE

There is an abundant literature relating to the effects of alcohol on the offspring, though little of it is scientifically reliable. I have attempted to select those cases which seem most trustworthy. Since we are more interested in the general problem of the effects of parental poisoning on the germ-cells and the embryo in mammals I have also collected the works relating to injurious substances other than alcohol. The observations and statistics on human beings in various countries are reliable only in so far as they may be substantiated and borne out by controlled experiments on lower animals. Yet in the light of animal experiments many of these human records become of surprising interest, although few if any of them may be accepted entirely as they stand.[1]

EFFECT ON THE MALE GERM-CELLS

It is a well known and universally accepted fact that alcohol does cause changes and degeneration in many of the body tissues of man. The question naturally presents itself, How, then, can the reproductive tissues escape? Nicloux and Renault have shown that alcohol has a decided affinity for the reproductive glands. In the testicular tissues and the seminal fluid an amount of alcohol is soon present which almost equals that in the blood of a person having recently taken alcohol. The proportion of alcohol in the testis as compared with that in the blood was as 2 to 3, and in the ovary of female mammals as 3 to 5. The genital glands show as great an affinity for this substance as does the nervous system. From these observations it must necessarily follow that alcohol may act on the ripe spermatozoon shortly before the time when it fertilizes the egg, and since an affected spermatozoon gives rise to a defective individual we have a probable explanation for many of the recorded defects attributed to drunkenness at the time of conception. A male, even for the first time, in a state of acute intoxication, is probably more apt to beget an abnormal offspring by fertilizing an egg at this particular period than is a non-intoxicated male although a frequent user of alcohol. The experimental data on the sensitiveness of the spermatozoon and the observations on the presence of alcohol in the seminal fluid warrant this statement.

1. Most of the literature is devoted to considerations of disease and insanity statistics and the family records of degenerates. The data are often collected in a careless fashion so that the *actual observations* are not always scientifically correct though the records are carefully and fully computed.

Lippich claims to have observed ninety-seven children resulting from such conceptions. Only fourteen of these were without noticeable defects. Eighty-three of them showed various abnormal conditions, twenty-eight were scrofulous,[2] three had "weak lungs," three showed different atrophic conditions, one watery brain, four were feeble-minded, etc. Others have made similar observations. Sullivan reported seven cases of drunkenness during conception which are fairly authentic. Six of the offspring died in convulsions after a few months, and the seventh was still-born.

Thus one finds proof by Nicloux and Renault that alcohol does reach the reproductive glands and, therefore, may affect the egg or sperm-cell, and observations seem to indicate that this effect expresses itself in the condition of the resulting offspring. Experiments on lower animals support the probability. When the perfectly normal spermatozoa of frogs are treated with *x*-ray or radium, Bardeen and O. Hertwig have shown that normal eggs fertilized by such spermatozoa all develop abnormally. Todde found that the offspring from alcoholized roosters were not quite normal and that the roosters did not succeed as well as normally in fertilizing eggs.

Combemale, 1888, was the first to experiment on the influence of alcohol on the mammalian offspring. He treated a dog for eight months with absinthe (11 gr. per day per kilo of animal weight) and paired this alcoholized dog with a normal bitch. Twelve young resulted; two were born dead, three died within fourteen days and the others died between thirty-two and sixty-seven days of intestinal catarrh, tuberculosis, etc. In a second experiment both parents were mated while normal, then the female was made drunk for twenty-three days (2.75 to 5 gr. absinthe of 72 per cent. per day per kilo). Of six young three were still-born, two had normal bodies though of weak intelligence, while one moved slowly and was very stupid. The last individual, a female, was later paired with a normal intelligent non-alcoholic dog. She gave only three young; one was deformed, club-footed with abnormal teeth, the second had a patent ductus arteriosis and died after fourteen days, while the third was poorly muscled in the hinder parts and died a few hours after birth. Thus the effects in the second generation are as pronounced as in the first although neither parent had themselves received any alcohol. The only criticism against Combemale's experiments is that an insufficient number of animals was used. Dogs often give defective pups and these may have been from poor stock, though such an interpretation is really not probable, and his results are supported by subsequent workers.

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2. Imbault, F.: Contribution a l'étude de la fréquence de la tuberculose chez les alcooliques. Thèse de Paris, 1901. Imbault found that tuberculosis was about as common among the children of alcoholic parentage as among those of tuberculous parents.

Hodge, in 1897, obtained similar results. From one pair of alcoholic dogs he obtained twenty-three pups, eight were deformed and nine were born dead, while only four lived. In a control set forty-one individuals lived, four were deformed and there were no still-births.

Laitinen treated rabbits and guinea-pigs with various doses of alcohol and studied chiefly the changes in body conditions as to resistance against disease toxins, etc. He has also recorded observations on the offspring produced by these animals during the experiment. He is apparently more interested in the problem of the misuse of alcohol than in the scientific study of the influence of injurious substances on the offspring and in his enthusiasm to prove the point with extremely small doses of alcohol he fails to fully consider both sides of his own tables.

He used daily doses of alcohol as small as 0.1 c.c. per kilo of animal weight. This would amount to a small glass (200 c.c.) of beer per day for an adult man. His tables on careful study fail to show that so little alcohol actually does injure the offspring of the treated animals.

With alcoholized rabbits Laitinen finds that only 38.71 per cent. of the young live, while 61.29 per cent. are still-born or die shortly after birth. In the control, however, only 45.83 per cent. lived, while 54.17 per cent., more than half, were still-born or died shortly after birth. The animals were kept all together in a general cage and the pregnant females were only separated shortly before the young reached term. This is scarcely an approved method in breeding experiments, and the fact that young rabbits are so delicate and are born in a rather poorly developed state makes their careful handling necessary. The fact that more than half of the control young die, 54.17 per cent., would indicate the danger of drawing conclusions from a death-rate only 7 per cent. higher among the offspring of the treated animals.

The case of the guinea-pigs is also indifferent, 78.26 per cent. of the control young lived, while 21.75 per cent., or a little more than one-fifth, of them died. The large majority of the young of treated parents also lived, 63.24 per cent., while 37.76 per cent. died. In both sets more of the young lived than died. Guinea-pigs are easily reared and are born in a well-developed condition. On the other hand, in both of the rabbit sets more of the young died than lived.

Results which I shall record below show that larger doses of alcohol do produce definite effects on the offspring. My experiments have been performed in a different manner and from another point of view. The primary object has been to regulate or control the type of development in mammals in a definite fashion as I had succeeded in doing with lower vertebrates. In these experiments it will be demonstrated that an alcoholized male guinea-pig almost invariably begets a defective offspring even when bred to a vigorous normal female.

Rösch was the first to study the reproductive glands of alcoholics, in 1837, and found degeneration of the testicles. Lancereaux described a parenchymatous degeneration of the seminal canals. Simmonds (1898) found azoospermia in 60 per cent. of cases of chronic alcoholism; 5 per cent. of these men were sterile. Kyrle reported three cases of total atrophy of the testicular parenchyma in which death had resulted from cirrhosis of the liver due to alcohol. Kyrle attributed the atrophy of the testicle to the cirrhosis of the liver and not to chronic alcoholism.

Bertholet (1909) made an extensive examination of the influence of alcohol on the histological structure of the germ glands, more particularly on the testicles of chronic alcoholics. He found testicular atrophy in alcoholics with no cirrhosis of the liver. Bertholet observed partial atrophy of the testicles in the majority of seventy-five chronic alcoholics. These men died between the ages of 24 and 57 years, the greatest mortality being between 30 and 50 years. In thirty-seven cases, excluding syphilitics, a microscopical examination showed a more or less diffuse atrophy of the testicular parenchyma and a sclerosis of the interstitial connective tissue. The testicles were small and hard. The canals were greatly reduced in size and their lumina obliterated. Spermatogonia were atrophic. It was generally impossible to differentiate spermatocytes or spermatids. There were no dividing cells and no spermatozoa. The thick basal membrane of the canals was formed of connective tissue lamellæ with concentrated spindle cells. These conditions with slight variations were found in twenty-four cases. Such atrophic structures were already present in a drinker only 29 years old. In four cases of cirrhosis of the liver the testicular atrophy had not progressed very far and spermatozoa were still present. In five cases the microscopical conditions were less marked.

While these appearances of the basal membrane may also be observed in non-alcoholics, the extreme conditions of atrophy of the testicles were only found in alcoholics. Observing the testicles of non-alcoholics that had died of various chronic illnesses such as tuberculosis, no atrophy of the testicles or thickening of the membrana propria was found. Two such old men of 70 and 91 years still possessed spermatozoa in the canals. Bertholet concludes that the atrophy he has observed cannot be due to old age, but is due to the hurtful effects of chronic alcoholism on the reproductive glands.

Bertholet has also reported an atrophy of the ovary and ova in female alcoholics. Weichselbaum has confirmed the observations of Bertholet at his institute in Vienna.

Bertholet's observations are most important and his drawings bear out his statements. On the other hand, it is certain that the chronic alcoholic is not so often rendered sterile as his study might lead one to

believe. Judging from the statistics it is not rare to find alcoholics with large families. My experiments on animals may not be of sufficient duration at the present time, yet I have male guinea-pigs that have been almost intoxicated on alcohol once per day for six days a week extending over a period of nineteen months. These animals are still splendid breeders. Nineteen months of a guinea-pig's existence is proportionally equal to a good fraction of a human life. Many of these animals have been killed and their testicles examined microscopically and found to be normal. In some cases where a male had failed to succeed in impregnating the female for several times, he was partially castrated, one testicle being taken out. In this case the testicle was found to be normal and the same male has since given offspring by other females. Ovaries have been examined in a similar way, and in no individual has the alcohol treatment caused a visible structural change in the reproductive glands. The actual physiological proof of the efficiency of the organs is shown by the ability of all animals to reproduce. The important point which I shall show in the following pages is that although there is no visible structural change in the germ-cells, nevertheless, they have been modified chemically to an extent sufficient to cause them to give rise to defective embryos or weakened individuals which die shortly after birth.

Schweighofer has recorded an interesting individual case. A normal woman married a normal man and had three sound children. The husband died and she married a drunkard and gave birth to three other children; one of these became a drunkard, one had infantilism, while the third was a social degenerate and drunkard. The first two of these children contracted tuberculosis, which had never before been in the family. The woman married a third time and by this sober husband she again produced sound children. This is an important human experiment. The female was first tested with a normal male and gave normal offspring; when mated with an alcoholic male the progeny were defective as a result of his poisoned condition. She was again tested with a normal male and found to be still capable of giving sound offspring. A number of such cases are on record.

Schweighofer states from a mass of observations that the offspring of drunkards, themselves of good sound families, show much degeneracy and defective conditions.

Other substances than alcohol seem to act directly on the germ-cells of mammals. Constantine Paul long ago pointed out that the children of people working in lead were often defective. He made the interesting observation that when the father alone was employed in such work his children were affected by it.

All of the above experiments and observations refer more particularly to the action of injurious substances on the germ-cells of the male parent.

This is the crucial proof of an effect on the germ cells. The case of the female is complex, since the substance may produce a germinal defect by acting on the egg, or it may also directly affect the developing embryo and thus act as an environmental influence on development.

THE FEMALE GERM-CELLS AND THE DEVELOPING EMBRYO

Herbst's classical lithium experiments show the influence of salt solutions on developing eggs. The experiments of J. Loeb on fish embryos, those of Morgan on the frog and my experiments on fish all show the marked influence of inorganic salts and organic compounds on the development of the embryo. I showed that alcohol caused all known

TABLE 1.—EFFECTS OF WORKING IN LEAD

	No. of Cases	No. of Pregnancies	Abortions, Premature Labor, Still-Births	Living Births	Remarks
Females showing lead poisoning symptoms..	4	15	13	2	One of the living children died in 24 hours
Females working in type foundry; previously had normal pregnancies	5	36	20	7	Four died in first year
Female in type foundry; five pregnancies	1	5	5	0	.....
Females working intermittently; while there	3	3	3	0	After being away for some time had healthy children
Females with blue line on gums, only sign of poisoning	6	29	21	8	.....
Male alone exposed.....	?	32	12	20	8 died first year, 4 second year, 5 third year
Total.....	..	120	83	37	22 died under three years

gross abnormalities of the brain in fish embryos and also gave all possible abnormal conditions of the eyes. Other substances such as ether, chloroform, chlorbutanol (chloretone), etc., also had a peculiar affinity for the developing central nervous system. These substances also act physiologically on the central nervous system of the adult.

Constantine Paul not only showed the injurious effects of lead on the paternal germ-cells, but also recorded instructive data regarding the offspring of women working in lead. More recent observers have pointed out the frequency of idiocy and other defects among the children of lead workers. Adami has tabulated the findings of Constantine Paul as shown in Table 1.

Forel states that acute alcoholic intoxication affects not only the brain, but, as Nicloux has shown, the alcohol passes quickly to the cells of the testicle or ovary and Bertholet's observations confirm this. A conception which takes place while the cells are in this poisoned state often results in a feeble-minded or degenerate child. The facts furnished by experiments on the eggs and spermatozoa of lower animals lend the strongest support to this idea and there is no experimental evidence that can be interpreted as opposed to Forel's statement.

Chronic alcoholics who consume daily certain amounts of alcohol slowly injure their germ cells. By intensive use of alcohol these cells may actually be killed or caused to atrophy. This, however, is the extreme case and before reaching a state of atrophy the cells pass through various grades of defectiveness. The stages may show no anatomic changes, but their physiologic state is indicated by the defective individuals to which they give rise in development.

Bezzola found that in Switzerland, in the years 1880 to 1890, there were 8,190 idiots. Most of the idiots were born in wine districts, and the season for the maximum birth of such children was nine months after the great national feasts, indicating, possibly, that idiots were conceived during the period of heaviest drinking. Schweighofer found the same relationship between the season for the greatest number of still-births and the feast seasons in Austria.

Martin studied the family histories of eighty-three epileptic girls in the Salpetrière (Paris). Sixty had alcoholic parents, while of the other twenty-three alcoholism was doubtful or absent in their parents. The sixty girls from alcoholic parentage had 244 sisters, of them 132, or 54.1 per cent., were dead; forty-eight, or 19.7 per cent., had had spasms during childhood.

Studying the direct ancestry of 370 insane people, Jenny Koller (in 1895) found that there were twice as many drinkers as were found in the direct ancestry of 370 sound people selected at random. Others have recorded similar observations.

Karl Pearson and Miss Elderson studied statistically 3,000 school children in England. They concluded that the children of alcoholics were often heavier than those of sober parents, they were also less diseased, had little epilepsy and tuberculosis and are actually cleverer in school. They found, however, a greater mortality among the children of alcoholics, especially of female drinkers, and concluded that only the stronger children lived, and therefore, their quality was good.

These studies have been widely criticised, and are probably not based on very thorough biological observations. They consider, in the first place, only school children. It is not known whether the parents were drunkards at the time of, or previous to the conception. The degenerate

offspring of alcoholics could not enter school. The results would doubtless have been quite different if the inmates of an institution for defective children had been studied. The great body of evidence from anatomic studies of the reproductive glands of alcoholics, the animal experiments and disease records are all opposed to Pearson's conclusions.

The most valuable study that I have been able to find on the influence of alcohol on the human offspring is that of Sullivan in 1899.

Sullivan emphasizes the point that while much effort has been made to record alcoholism in the ancestry of degenerates, the important study must be made on degeneracy in the descendants of well-observed alcoholics. He studied the alcoholics among the female population of the Liverpool prison and as far as possible chose cases of alcoholism that were unaccompanied by disease or other degenerate factors.

Localization of alcoholic lesions in the body are not well worked out, yet it is unquestionable that in the criminal, as in insane alcoholics, the nervous manifestations of the intoxication occur with notable frequency, while non-nervous disorders are rare or secondary. Of these alcoholic females, thirty-one had had one or more attacks of alcoholic delirium, twenty-four had occasional hallucinations, suicidal impulses; disorders of cutaneous sensibility, and cramp in the extremities was noted in a considerable number of cases. In these patients tissues other than the nervous, so far as examination of the patients themselves could show, were comparatively immune to the poison of alcohol, and this was also true of their alcoholic relatives.

There were 100 women in the series Sullivan observed, and twenty of these gave details of female relatives of drunken habits who had children. To these 120 females were born 600 children, of whom 265, or 44.2 per cent., lived over two years; 335, or 55.8 per cent., died under 2 years or were still-born. Twenty-one of the women observed gave records of sober relatives, sisters or daughters married to sober men. The twenty-one drunken females had 125 children, sixty-nine, or 55.2 per cent., died under 2 years; the twenty-eight sober females had 138 children, and thirty-three, or 23.9 per cent., of them died under 2 years. The death-rate of children from the drunken mothers was nearly two and one-half times greater than that of the children of their near-blood relatives who were non-alcoholic. The alcoholics, however, are poor mothers and take little care of their children; this fact might possibly account for the entire difference, though such a deduction is extremely improbable.

The progressive births in the alcoholic family show interesting records. In eighty cases the number of children reached or exceeded three.

The tabulation shows an increasingly poor condition. The records of two individual cases may be mentioned by way of illustration:

Case 5: Three first children healthy, fourth of weak intelligence, fifth epileptic idiot, sixth still-born, and finally an abortion.

Case 10: First child survived to adult life, second died of infection as child, two infants then died in convulsions in first few months, then a still-birth.

These records stand in interesting contrast with those known for syphilitic mothers in which each conception seems to be more and more nearly successful until a weak offspring is born, and finally such a mother may give birth to an apparently normal child. The syphilitic is gradually becoming less diseased and is overcoming the toxic condition as time goes on, while these alcoholic women are on the contrary becoming more and more saturated with the poison, and for this reason each succeeding birth is more decidedly defective.

TABLE 2.—SHOWING PERCENTAGE OF STILL-BORN AND CHILDREN WHO DIED IN AN ALCOHOLIC FAMILY

	Cases	Died or Still-Born, Per cent.	Still-Born, Per cent.
First born	80	33.7	6.2
Second born	80	50.0	11.2
Third born	80	52.6	7.6
Fourth and fifth born	111	65.7	10.8
Sixth to tenth born	93	72.0	17.2

The records were worse for women who had begun drinking some time previous to the first conception. In thirty-one cases they had been drinking for at least two years before the first pregnancy. Of 118 children born to these, seventy-four were still-born or died in infancy, giving 62.7 per cent. as compared with a death-rate of 54.1 per cent. for the others of the series.

In only thirty-nine of the cases were the women's parents sober people, yet the records of the offspring from these women were equally as bad as those from the sixty-one mothers who had alcoholic parents. This is a significant fact, since it indicates most strongly that the defective children are due to the direct effect of alcoholism and not to other degenerate conditions. Sullivan recorded seven known cases of conception during a state of drunkenness; six of the children died in convulsions in a few months, while the seventh was still-born.

Another observation by Sullivan which indicates that the alcohol as such is the cause of defectiveness was the fact that mothers imprisoned during pregnancy gave birth to a better child since the drinking was stopped.

Sixty per cent. of the children of all these mothers died in convulsions. This is a common manner of death for the offspring from the alcoholic mammals I have studied.

Kende found that of twenty-one families in which the father and mother both drank, ten were childless, while of the twenty-four children in the other eleven families, sixteen died early and only three were entirely normal. In eighteen families in which only the father drank, but three children in twenty-one were entirely sound, while there were many abortions and several cases of sterility.

There are numerous statistical facts showing a large percentage of alcoholics in the ancestry of prostitutes, degenerates and other inferior classes. All of the studies seem to show that alcoholism and the degenerate condition tend to occur in the same family, and Sullivan seems to control the case by showing that in some instances, at least, alcohol is the cause of degeneracy.

The real, crucial proof of the direct action of alcohol must come, however, from experiments on lower animals, where the sources of error may be entirely controlled.

Adami states: "The general belief (and we regard it as well founded) is that the children of the sot are as a body of lowered intelligence and vitality with unstable self-control." He recognizes the great difficulty of statistically proving this in man, since alcoholism is so often the accompaniment of weakness and hereditary taint, and may not be the primary cause of the condition in many families. With animals, however, the experimenter is enabled to prove that alcohol does induce a primarily degenerate condition.

One could continue to enumerate records showing the effect of alcoholism on the human offspring, yet a sufficient number of studies have been considered to show how strongly indicative the evidence is that alcohol is really the direct cause of defects in many cases. There is also little doubt that alcoholism is sometimes acquired by perfectly normal human beings, and when the tissues of such people become affected by alcohol they no doubt give rise to defective and abnormal offspring.

It is, however, an undeniable fact that alcoholism in man is very frequently an accompaniment of various degenerate conditions, and these conditions are oftentimes within themselves sufficient to account for further degeneration in the offspring. We shall, therefore, consider more fully at this point the evidence furnished by animal experimentation.

ANIMAL EXPERIMENTS

As stated above, the problem is broader than the subject of alcoholism. If it is shown that any toxic substance can act on the germ cells or developing embryo in such a manner as to change or modify its

development, it necessarily follows that alcohol may induce a more or less equivalent condition, since it is definitely known to act on all animal tissues. I shall, therefore, mention the experiments with alcohol in particular, and at the same time consider other of the striking examples of environmental effects on the developing eggs of lower animals.

H. E. Ziegler treated sea-urchin's eggs with ethyl-alcohol. A 1 per cent. solution in sea-water delayed development, a 2 per cent. solution also delayed development and caused abnormal embryos, while a 4 per cent. solution prevented all development. The peculiarly typical larvæ of the sea-urchin which Herbst induced by the addition of lithium salts to sea-water have been mentioned above. Herbst's experiments furnish a striking example of a characteristic response on the part of the developing organism to a definite chemical treatment. Morgan obtained similarly definite results by treating frog's eggs with lithium, and I have shown a somewhat comparable response for the fish's egg. Other salts may give the same types of larvæ, as occurred in these cases, as McClendon has shown for the fish, and as I previously pointed out in several of my studies on the cyclopean defect. Yet with certain doses of given substances one gets greater numbers of the same defect than with any other treatment. It is not surprising that a few individuals of any one deformed type may occur in a number of different solutions. The important fact is, that with a particular treatment one is able to obtain on all occasions a large number of embryos exhibiting a perfectly clear-cut, definite defect.

Ridge got decided results by treating the eggs of the blue bottle-fly and frogs with alcohol. In solutions of 1/100 per cent. alcohol in water the development was slow. In 1/20 per cent. solutions development proceeded for only a short time and the eggs died. In one per cent. alcohol only one or two eggs started.

Ovize made an interesting observation on the influence of alcoholic fumes on developing hen's eggs. An incubator containing 160 eggs was in a cellar in which wine and brandy were being distilled. Seventy-eight chickens hatched; of these twenty-five were deformed and forty died during the first three or four days. Of the number unhatched, one-third were deformed, and 3 to 4 per cent. had only developed a short way.[3]

Féré has experimented extensively with the influence of alcohol on the developing hen's egg. Alcohol was injected into the albumen in some experiments, while in others the eggs were placed under bell jars and exposed to the fumes of evaporating alcohol. Enough of the fumes penetrated the shell and entered the egg to affect the subsequent development of the embryos. When eggs were placed in the incubator after such

3. These results by Ovize were taken from Forel's review.

treatment they developed more slowly than the control and a large number of malformed embryos resulted. The abnormalities were variable, yet many had defective nervous systems and a number of the embryos exhibited eye defects. Féré made no attempt to analyze the cause of the different types of deformities, and in fact he paid little attention to the structure of the defects. Yet he showed most decidedly that alcohol fumes do affect the developing embryo, as one might have inferred from the preceding observations made by Ovize.

I have repeated Féré's experiments at some length during the past two years and can confirm his results. My object has been to regulate the treatment in such a manner as to get definite types of defects with certain intensities of treatment. Up to the present time I have only partially succeeded in doing this, though in several experiments the delay in development and the general type of the defects has been rather constant. This treatment of hen's eggs with alcoholic fumes is one of the most convincing and easily performed demonstrations of the influence of alcohol on development.

Féré also experimented with hen's eggs to show the influence of differences in temperature during incubation and many other physical and chemical factors. All unusual conditions affected the development of the embryo. Féré also developed hen's eggs in glass dishes after removing them from the shell. Preyer and Loisel had previously done similar experiments, but they carried the embryo for only a day or so, while Féré succeeded in keeping the egg developing for six days. Some of these embryos develop abnormally.

I have recorded a number of experiments on fishes' eggs which show the decided effects of alcohol and a large series of other substances on embryonic development. Alcohol and various anesthetics showed a peculiar affinity for the developing nervous system and organs of special sense. In many cases other organs and parts of the embryos were apparently normal. Many of the deformed individuals hatched and lived for some time, swimming about and feeding in a typical fashion.

With alcohol solutions of given strength definite defects were induced. In some experiments dozens of embryos with typical brain and eye defects occurred, while few or no other types of deformities existed. *The experimenter has the power in these cases to predict with at least a limited degree of certainty the type of deformity which will result from a definite intensity of a particular treatment.* Embryonic development in such cases may really be regulated or controlled.

We have already considered a number of experiments on mammals which show that alcohol and other injurious substances affect the quality of the offspring. In treating mammals the case is not so simple as in treating the eggs of lower vertebrates which develop outside the

parent's body. The effects in mammals may not be due directly to the substance used, but rather indirectly to the changed conditions the substances have induced in the body of the parent. It is important for this reason to know whether certain substances come in direct contact with the germ cells of the individual. As before mentioned, Nicloux and Renault have shown that alcohol may be readily found in the seminal fluid of a man shortly after drinking it. Thus the spermatozoa may come to float or swim in a weak solution of alcohol.

In the case of female mammals, Nicloux has carefully demonstrated the passage of alcohol from the blood of the mother into the tissues of the embryo. The following tabulation readily shows the results of his experiments on dogs and guinea-pigs:

TABLE 3.—PASSAGE OF ALCOHOL FROM THE MOTHER TO THE FETUS

		Amt. of Abs. Alc. Inject. per Kilo of Animal Weight, c.c.	Time of Absorption; Animal Killed, Hours	Amt. Alc. per 100 c.c. Maternal Blood, c.c.	Amt. Alc. per 100 c.c. Fetal Blood, c.c.	Amt. Alc. per 100 gm. Mother's Liver, gm.	Amt. Alc. per 100 gm. Fetal Tissue, c.c.
1.	Guinea-pig	5	5/6	0.36	0.31	... .	.....
2.	Guinea-pig	5	1	0.47	0.35	.....	.....
3.	Guinea-pig	2	1	0.20	....	0.10	0.12
4.	Guinea-pig	1	1	0.13	....	0.081	0.086
5.	Guinea-pig	0.5	1¼	0.045	....	0.015	0.02
6.	Dog	3	1½	0.37	0.37	0.26	0.26

After a short period of time the amount of alcohol in the blood of the fetus is about equal to that in the blood of the mother, while there is really more alcohol in a given weight of the tissues of the fetus than is to be found in an equal weight of liver tissues from the mother.

The reality of the passage of alcohol from the mother to the fetus demonstrates the possibility of the intoxication of the fetus. Therefore, nervous disorders, anesthesia, etc., of the late fetus may result as a consequence of alcohol in the blood, while the developing embryo or early fetus will show the effects by an abnormal formation of the nervous system.

Thus the results of the experiments of Mairet, Combemale and Hodge on dogs are readily explained as the direct influence of alcohol on the paternal germ cells in the case of the treated male, or on the developing fetus within the body of the alcoholic mother. The great number of human records briefly referred to above are also readily interpreted as the result of direct alcoholic action on the germ cells and the developing embryo.

The experiments on mammals do not then really differ greatly from those on the lower vertebrates where the externally developing eggs are placed directly in various unusual solutions, since the egg or embryo although within the mother's body is readily bathed or impregnated by the alcohol contained within the mother's blood.

The only experiments with alcohol on lower mammals which do not fall completely in line with the above records are those recently recorded by Nice. He has fed mice on alcohol. Each day 2 c.c. of 35 per cent. alcohol was added to crackers and milk and placed as food for each mouse. Instead of drinking water the mice could drink 35 per cent. alcohol from a syphon which prevented evaporation. Animals treated in this way gained in weight over the control. The offspring from these alcoholic mice excelled all the other mice in growth, even when they themselves were fed alcohol. The young grew faster, however, when not given alcohol. Nice treated other mice with tobacco fumes, nicotin and caffein. The fecundity of the alcohol, nicotin and caffein mice was greater than the control while those treated with tobacco fumes had almost twice as many young as the control. The mortality of the offspring from the treated mice was, however, greater than from the control. None of the control young died, while 17.3 per cent. of the nicotin young and 11.1 per cent. of the alcoholic young died soon after birth. There was only one abortion, no still-births and none of the young were deformed.

Mice may possibly be peculiarly resistant to these drugs, though I should rather think that in the case of alcohol, at least, the animals received too little to give a pronounced effect, though it was sufficient to cause a certain fatality among the young. Weak alcohol mixed with crackers and milk no doubt rapidly evaporates. The animals possibly waited until a certain amount of the alcohol had disappeared before they ate their food, and, of course, the amount of alcohol they took instead of drinking water was very small. Mice may easily be kept on a cracker and milk diet without ever receiving water. One cannot deny, however, that the mice did receive enough alcohol to cause them to fatten more rapidly than the control, and probably to cause the death of some of their offspring.

Carrière has shown that when guinea-pigs are inoculated with various soluble products of the tubercle bacillus for several months that the number of offspring is diminished. He sometimes observed the death of the fetus or premature death of the young, while many of the living young had feeble constitutions. The action was produced when either parent was impregnated with the poison.

Mating together two inoculated animals gave 52 per cent still-born young; 28 per cent. of the living young died under sixteen days and

only 20 per cent. of the young survived. When the female alone was inoculated 26.9 per cent. of the offspring were still-born, 34.6 per cent. died under sixteen days and 38.4 per cent. of the young survived. The matings with the male alone inoculated gave 16.6 per cent. still-born, 10 per cent. dying under sixteen days and 73 per cent. of the offspring survived. Thus the effect of the toxin is shown on the germ cells of both sexes.

Lustig's experiments of inoculating fowls with abrin gave results parallel to those recorded by Carrière. The offspring were less resistant to inoculations of abrin, just as the guinea-pigs were to the tuberculosis extracts when compared with control animals of the same age.

Mall has clearly shown in his monograph on the causes of human monstrosities that poor nutrition and abnormal environment are most potent factors. Only 7 per cent. of the uterine pregnancies examined gave monsters, while 96 per cent. of the tubal pregnancies produced abnormal embryos.

Ballantyne has presented in his "Antenatal Pathology" a most comprehensive consideration of the part played by abnormal environment and disease in the causation of monstrosities and developmental defects in general.

The effects of malnutrition or poor environment on the developing embryo is splendidly illustrated by the case of monochorial twins when one becomes more vigorous and pumps blood from the other through the anastomoses between their placental or umbilical vessels. In such cases one of the twins may fail to develop certain parts and may actually lack a heart, the heart of the superior embryo pumping blood through both the bodies. The various degrees of the degenerate or parasitic twin is thus produced. One individual falls behind in development and may finally actually be included within the body of the more vigorous twin. Double monsters may occur in which one individual is almost perfect, while the smaller monster is attached to some part of its body.

I have given this somewhat extensive survey of the literature in order to show that an abundance of evidence exists at the present time to indicate that the course of embryonic development may be readily modified. It is also clearly shown that the germ cells of various animals may be directly affected by different chemical treatments to such a degree that they give rise to defective individuals. The experiments of O. Hertwig and Morgan on the chemical production of spina bifida in large numbers of tadpoles and my experiments on the constant production of typical cyclopian monsters by subjecting developing fish eggs to definite chemical treatments strongly indicate that the manner of embryonic development may be definitely regulated. This exact regulation or control of development is the important goal of experimental teratology.

The problem is now in its beginning, since the actual influence of various treatments is known to be expressed in the resulting type of embryonic development.

The studies on alcoholism in mammals have failed to produce any convincing evidence of the specific actions of this poison. Yet the statistical studies on defective human beings would indicate that alcohol had a special affinity for the developing nervous system. My experiments on the influence of alcohol on the developing fish embryo demonstrated that alcohol did have a specific affinity for the central nervous system, and caused the brains of these embryos to exhibit numerous deformities, while the organs of special sense were also affected.

METHOD AND RESULT

The experiments here recorded have been undertaken in order to ascertain whether alcohol did exert a marked influence on the germ cells and developing embryos of mammals, and, if possible to demonstrate the nature and mode of action of this influence. I have used alcohol as an agent, since it may be given to guinea-pigs without greatly disturbing their normal physiological processes, and so does not produce marked conditions which might secondarily affect the results. Alcohol may remain as such in the blood and tissues of a mammal, and so may act directly just as it would when added to the sea-water in which fishes' eggs were developing. I have studied its effects experimentally on the eggs of lower vertebrates and am familiar with the defects it produces in these animals. It is an active substance and, therefore, for these many reasons lends itself admirably to experimental use.

The experiments have been conducted on guinea-pigs, since they breed fairly rapidly and rear their young without much difficulty in the laboratory. Strong healthy stock has been chosen and the animals have been carefully handled. All have remained in vigorous health and most of them have increased in size and fattened during the progress of the experiment. The males and females have been kept carefully separated and individual pairs mated from time to time.

The animals are first tested by normal matings and found to produce normal offspring. The alcoholic treatment is then begun on a given number of individuals and males and females mated in different combinations according to whether they are alcoholics or normal. An alcoholic male is mated with a normal female, the paternal test. This is the crucial test for influence on the germ cells, as here the defective offspring must be due to the chemically modified spermatozoon from which it arose, since the egg, and the mother in which the embryo developed, were both normal.

Normal untreated males are paired with alcoholic females, the maternal test. Here the defective offspring may be due either to a modified ovum or to the fact that it developed in a mother with alcoholic blood, therefore supplying an unfavorable developmental environment. Lastly, its condition may be due to both of these causes. The mammalian mother has two chances to injure an offspring, either by producing a defective egg, or secondly by supplying an unfavorable or diseased environment in which the embryo must develop.

The final combination is the mating of alcoholic individuals. This, of course, offers the greatest chance for defective offspring.

Alcohol is administered to the guinea-pigs by inhalation. At first it was given with the food, but the animals did not relish it, and therefore took less food. It was then given by stomach tube, but this method

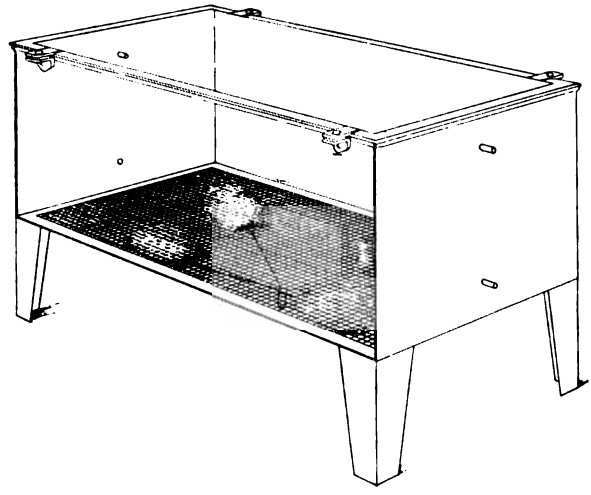

Fig. 1.—Tank for alcohol treatment. Animals are placed on the wire screen in the closed tank and inhale the fumes of alcohol evaporating from the cotton below the screen.

so upset the animals that the results might have been modified by their poor bodily condition and the bad state of their stomachs. The inhalation method is entirely satisfactory, the guinea-pigs thrive and usually gain in weight during the experiment, they have good appetites and are in all respects apparently normal. The only indication of the effects of the treatment is shown by the quality of offspring they produce.

The apparatus used for giving the alcohol consists of an air-tight copper tank 36 inches long by 18 inches wide and 12 inches deep, with a sloping bottom draining to the center. Over this bottom is placed a wire screen and below the screen cotton soaked with 95 per cent. alcohol is spread (Fig. 1). The tank is closed and allowed to stand until the

atmosphere within is saturated with alcoholic fumes. A ventilation system is so arranged that a given quantity of alcohol fumes may be driven through the tank in a given time, but it has not seemed advisable to use this device, as the degree of intoxication is a better index to the physiological response of the animals, since their resistance to a given amount of the fumes is changeable. The guinea-pigs, three or four at a time, are placed on the wire screen above the evaporating alcohol, the tank is again closed and the animals are allowed to remain until they begin to show signs of intoxication, though they are never completely intoxicated. They usually inhale the fumes for about an hour. The animals are treated in this way for six days per week and some have now been treated over a period of about nineteen months. None of the effects

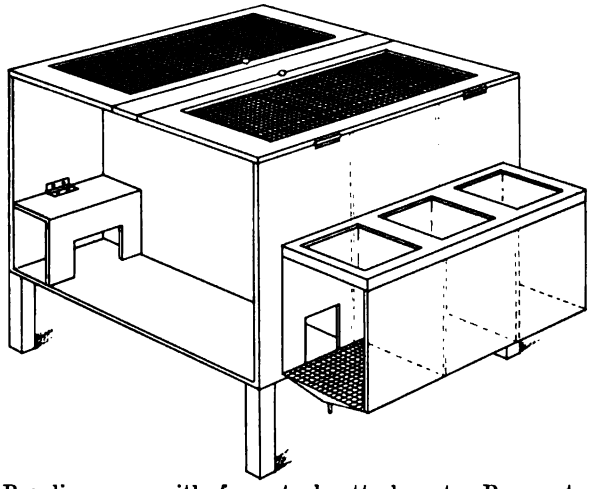

Fig. 2.—Breeding cage with fume tank attachment. Pregnant females are kept in this cage and may be driven through the drop door into the fume tank. Handling pregnant animals during the treatment is thus avoided.

are due to want of air, since the same number of guinea-pigs may remain for hours in this closed tank without showing any signs of discomfort when there are no fumes present.

In order to avoid handling the females during late pregnancy, a special treating cage is devised for them. An ordinary box run with a covered nest in which the animal lives is connected by a drop door with a metal-lined tank having a similar screen arrangement to that described for the general treatment tank (Fig. 2). The pregnant animal may be driven daily into the tank and thus treated with alcohol fumes throughout her pregnancy without having to be handled or moved about in any way that would tend to disturb the developing fetus.

During the vapor treatment the animals usually react in a manner quite similar to their behavior in weak fumes of ether or chloroform.

The majority of them sit quite motionless and sniff their noses for a time and then become somewhat drowsy. A few individuals, however, are excited by the treatment and run about the tank, becoming sexually excited, and many often fight other animals savagely. One of the males fights and bites so vigorously while taking the fumes that he has to be treated separately from all others. The fumes then have a different influence on the behavior of different individuals in much the same way that alcoholic intoxication expresses itself differently on different human beings.

During the first few weeks of the treatment the fumes cause the eyes to water so that tears run over the face. The nose and mouth also become moist and the animals sniff almost constantly. The fumes are very irritating to the mucous membranes at first. The cornea becomes irritated and finally opaque in some instances, so that the eye takes on a white appearance. The tissues seem, however, to develop a resistance to the fumes. The eyes become clear after a few months and never again become opaque. The nasal mucosa also ceases to secrete excessively unless the animal is left in for an unusually long time.

Many of the guinea-pigs have been killed after treatments of different duration up to fifteen months, and all of their viscera carefully examined. In no case have I found any changed structures due to the alcoholic treatment. The lungs, liver, stomach, intestines, kidneys, reproductive glands, brain and all other parts appear perfectly normal. The general health and behavior of the animals also indicate that they are in good condition. As before mentioned, several animals have been partially castrated during the experiment. One of the reproductive glands was removed and examined microscopically. In all cases the germ cells, ova or spermatozoa, as the case may be, were found to exhibit perfectly normal structure. One cannot claim, therefore, that this treatment is excessively severe or greater in proportional amount than the alcohol a human being often takes. The fact is that these animals have never been completely intoxicated, but receive only enough alcohol six times per week to affect their nervous states. They may be compared to a toper who drinks daily but never becomes really drunk.

While the bodies of these animals display no direct effects of the alcohol, the conditions of the offspring to which they give rise show most strikingly the effects of the alcoholic treatment. The results of mating the alcoholized guinea-pigs are summarized in Table 4.

Fifty-five matings of treated animals have been made. Forty-two of these have now reached full term and are recorded. Thirteen matings are not yet due. From the forty-two matings only seven young survived, and six of these are still living, five of which are runts, though their parents were unusually large, strong animals (Figs. 4 and 5).

The conditions of the animals in the mating pairs are shown in the first column of the table and the total results of the matings are indicated in the following columns. The first horizontal line gives the records when alcoholic males are paired with normal females. Twenty-four such matings were made. Fourteen of these gave negative results, or resulted in early abortions. Many embryos were aborted during very young stages, and some of these were deformed, though they were generally in such poor condition after being cast out into the cages that little could be learned from them. They were partially or completely eaten by the mother in most cases. The males were always kept for a number of days with the females during favorable periods, and conception should have occurred in all cases, as it did in the control matings.

TABLE 4.—EFFECTS OF ALCOHOL ON OFFSPRING OF GUINEA-PIGS

Condition of Animal	No. of Matings	No Result or Early Abortion	Still-Born Litters	No. Still-Born Young	Living Litters	Young Dying Soon After Birth	Surviving Young
Alcoholic male by normal female	24	14	5	8	5	7	5*
Normal male by alcoholic female	4	1	0	0	3	3 (*a*)	2†
Alcoholic male by alcoholic female	14	10	3	6	1	1 (*b*)	0
Summary	42	25	8	14	9	11	7‡
Normal male by normal female—Control	9§	0	0	0	9	0	17

*Four survivors in one litter, and one was a member of a litter of three, the other two died immediately after birth. (*a*) Premature. (*b*) Sixth day.

†One lived to become pregnant with two young *in utero*, one deformed, Fig. 3. Other survivor normal, the mother was not treated until after first two or three weeks of pregnancy.

‡Of thirty-two young born only seven have survived.

§One other non-alcoholic mating was made from which two young resulted; they died after the second and fourth days, respectively, and the mother died two days later; her diseased condition no doubt affected the suckling young. They have for this reason not been included in the normal control.

Only ten of the twenty-four matings resulted in conceptions which ran the full term. Half of these, or five, were still-born litters. There were three still-born litters of two young each and two of one individual each. Most of these were slightly premature, their eyes being closed and the hair sparse on the bodies. (A normal guinea-pig at birth is well covered with a hairy coat, its eyes are open and it very quickly begins to run about actively.)

Five litters of living young were born. One litter consisted of only one young, a weak individual that grew very little and died after six weeks. Two litters contained two young each. The members of one of these litters died during the first and fourth weeks, having been weak and small since birth. Both of those in the other litter were in a similarly feeble condition and died before the first month. One litter contained three young; two of these died immediately after birth; the other one is still alive, though small for its age. The fifth litter contained four young, all of which are runts, though their parents were unusually large animals (Figs. 4 and 5). *Thus out of twenty-four full-term young, of which only twelve were born alive, but five individuals have survived, and these are unusually small and very shy and excitable animals.*

It is a point of some interest that all of the young animals that died showed various nervous disturbances, having epileptic-like seizures, and in every case died in a state of convulsion. This is commonly the fate of feeble and nervously defective children.

The important fact in the above case is that only the father was alcoholic, the mother being a normally vigorous animal. *This experiment clearly demonstrates that the paternal germ cells may be modified by chemical treatment to such a degree that the male will beget abnormal offspring even though he mate with a vigorous female.* A reconsideration of the figures in the first line of the table shows really how decidedly the injured spermatozoon expresses itself in the fate of the egg with which it combines.

The second line of the table shows the results of matings between alcoholized females and normal males. These matings might be expected to give more marked results than the pervious ones, since in the treated females not only the germ cells may be affected, but the developing embryo itself may be injured by the presence of alcohol in the blood of the mother. Nicloux has shown that alcohol may pass directly from maternal blood into the embryonic tissues of a guinea-pig. The spermatozoon, however, is probably a more sensitive structure than the egg and is easily injured or killed by slightly abnormal conditions. It might possibly be that when such a specialized cell swam for even a short period of time in seminal fluid containing a trace of alcohol its chemical nature would be so decidedly disturbed as to render it incapable of inducing normal development after impregnating the egg. At any rate the few cases at present available seem to indicate that the effect on the offspring is equally as great when it is produced by an alcoholic father as by an alcoholic mother.

There are only four matings between alcoholized females and normal males. One of these gave a negative result or was possibiy aborted very early. Three living litters were born. One of these consisted of three

premature young, which died shortly after birth. The remaining two litters each contained only one young, but these two animals survived. One of these guinea-pigs was born after the mother had been treated for three and one-half months. The offspring was weak and small for several months after birth, but finally recovered and developed into a normal animal. This guinea-pig was mated with an alcoholic male and became pregnant. Unfortunately, she was killed by accident, and on examination her uterus was found to contain two embryos, 33 and 32 mm. in length. One of these embryos was deformed and showed very decidedly

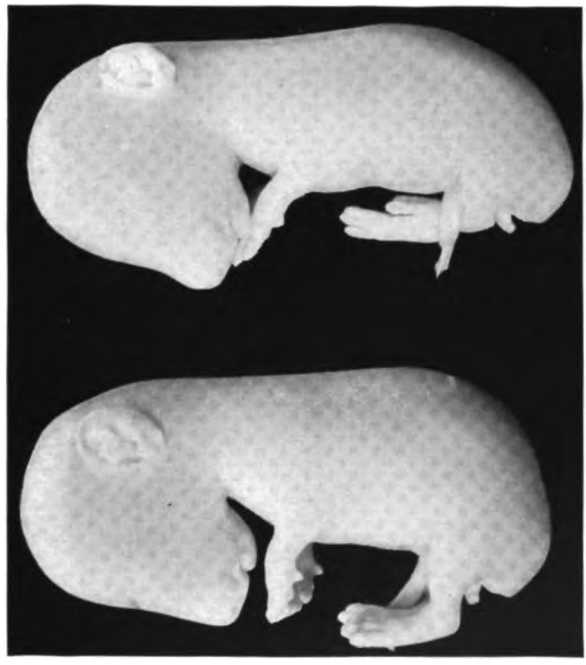

Fig. 3.—Two embryos 32 and 33 mm. in length, taken from a female that had an alcoholic mother and was mated with alcoholic male. The upper fetus has deformed hind legs and a poorly developed posterior part of the body; lower fetus is normal.

degenerate and feebly developed hind legs. The posterior end of its body was also poorly formed. This condition is readily seen in Figure 3, a photograph of the two embryos. The abnormal one has small hind legs, and one of them is badly folded under its body. This is of interest, since all of the affected offspring of alcoholic guinea-pigs are weak in their hind extremities and drag their legs. Yet none were so modified as to show a noticeable structural defect except this embryo, which had one alcoholic grandmother and an alcoholic father.

The only other survivor from an alcoholic mother is strong and full grown for its age. The mother had been treated for only two and one-half months when the offspring was born, so that she was normal during the first two or three weeks of pregnancy. No doubt the early stages of development are more easily modified to produce significant defects than are the later. This question is being more fully tested on guinea-pigs with experiments now in progress. I have shown, however, in

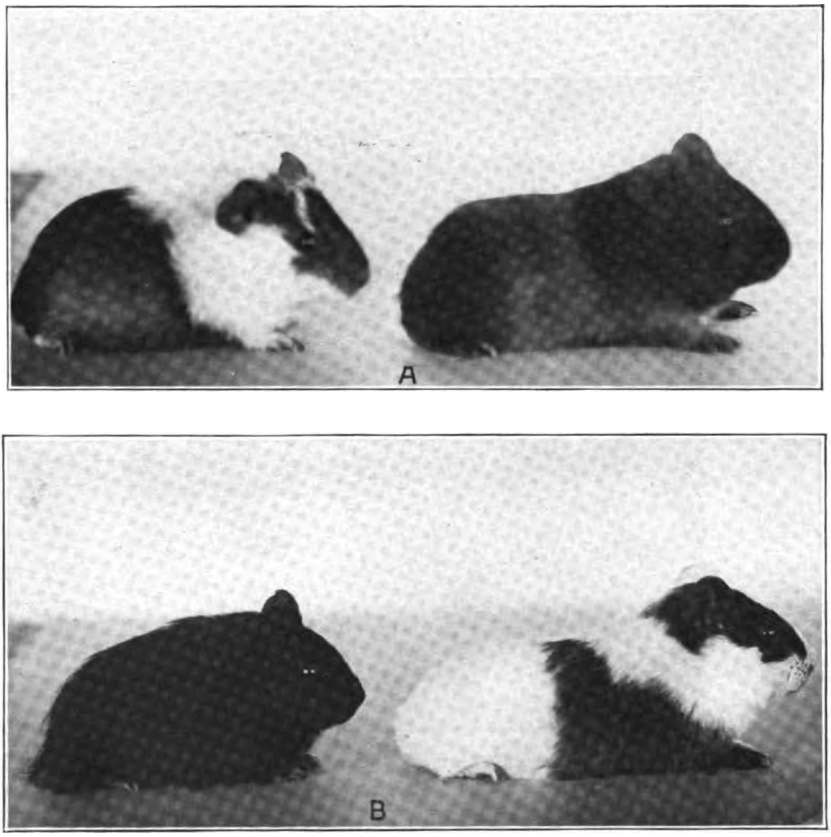

Fig. 4.—A. The animal on the left is a runt from a large alcoholic male and a large normal female; weighs 134 gm. The animal on the right, from normal parents, is larger although 1 month younger and weighs 147 gm.

B. The guinea-pig on the left is a runt, weighing 132 gm. from an alcoholic father; on the right a normal guinea-pig twice as large though only 10 days older, weighs 221 gm.

treating fish eggs that the period at which the treatment is applied is a most important factor in determining the type of defect or modification which will result. Certain salts, different strengths of magnesium chlorid, for example, which give pronounced effects when added to the

sea-water containing eggs in early developmental stages, may really be ineffective after the eggs have developed beyond these stages. In the case under consideration the offspring might not have fared so well if the alcoholic treatment had been started on the mother a few weeks before conception, instead of three weeks after her pregnancy had begun. This with other points shall be more completely analyzed in future communications on these experiments.

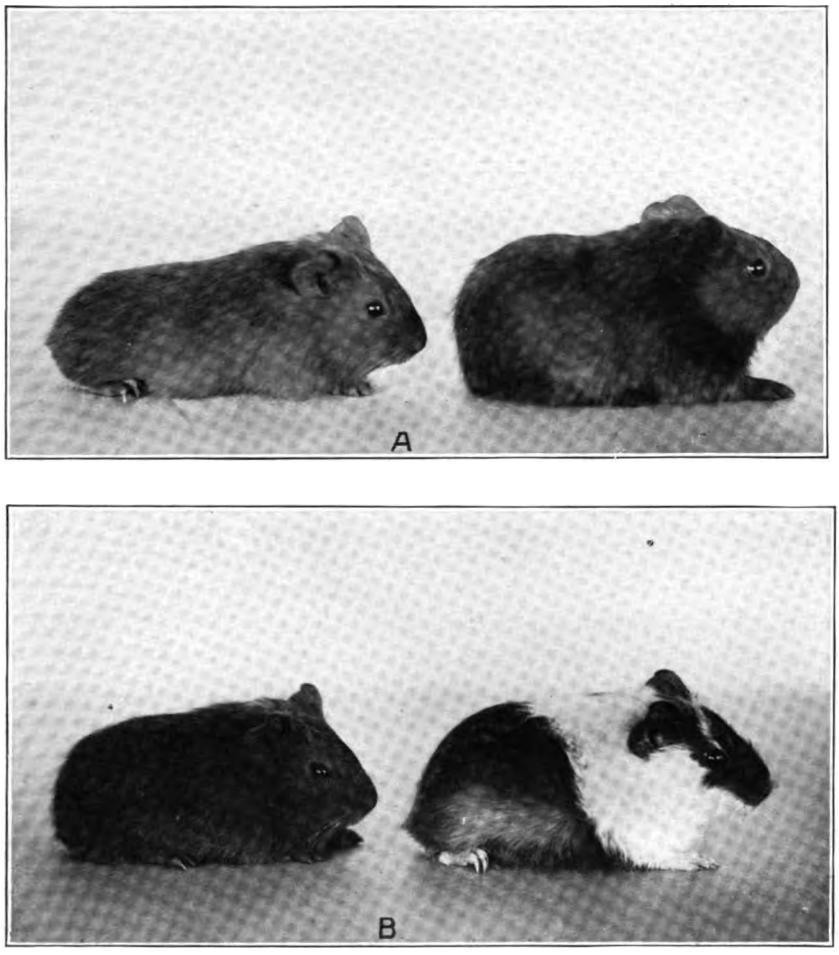

Fig. 5.—A. Two guinea-pigs from alcoholic fathers, the left one 1 month and 10 days younger than the runt on the right.

B. The left animal is the same as above, the right another of the same runt litter.

The four matings of alcoholic females and normal males resulted in three living litters in all of five individuals. Three of the young were premature and died shortly after birth, while two young survived.

Finally, we may consider the results of pairing two alcoholized individuals. The third line of the table summarizes these results. As might have been anticipated, this type of mating has given the highest fatality of all.

Ten out of a total of fourteen matings have given no offspring or early abortions, which were in many cases eaten by the mother. Three still-born litters have been produced, each consisting of two young. *Only one living litter was born from the fourteen matings in which both parents were alcoholic, and this litter consisted of but one weak individual which died in convulsions on the sixth day after birth.* This is indeed a decided effect of alcohol on the offspring when one compares it with nine control matings, all of which gave living litters containing a total of seventeen individuals, all surviving.

Two other young were produced by non-alcoholic parents and died on the second and fourth days after birth. They have not been included in the control since the mother died two days later in a diseased condition. No doubt the poor state of the mother had much to do with the fate of the suckling young. She was an animal that had only been in the experiment for a short time, and is one of the very few that have contracted disease or died during the nineteen months of the work. This might possibly go to show the influence of a diseased mother on the offspring.

The fourth line of Table 1 gives a summary of the experiments. There have been forty-two full-term matings, twenty-five of which gave no results or early abortions; eight still-born litters have occurred, consisting of fourteen individuals; only nine living litters have been born, 21 per cent. of the matings. These contained eighteen young, and but seven of this number have survived and five of these survivors are unusually small (Figs. 4, 5).

The bottom line of the table shows nine control matings. All have given living litters containing a total of seventeen young, all of them surviving. The two young that died, as stated above, were from a dying mother and not included in the control.

Records of the successive matings of ten of the female guinea-pigs are shown in Table 5. The varying ways in which the same individual has responded in different matings is noticeable. Number 10, an alcoholic female, first mated with an alcoholic male, gave one young which died on the sixth day after birth. On being remated with the same male, No. 10, gave no result. When mated with another alcoholic male, gave no result. She mated again after several months with the first male and on being killed was found to contain one embryo *in utero* about 2 weeks old.

Female 15, a normal guinea-pig, shows an instructive record. She was mated with an alcoholic male and gave birth to two still-born young.

When mated with another alcoholic male she gave a negative result. Remated with the second male she gave two young, both of which died of convulsions within four weeks after birth. She was then mated with a normal male as a control and gave one vigorous normal offspring which survived.

TABLE 5.—RESULTS OF SUCCESSIVE MATINGS OF TEN FEMALES

Animal	First Mating	Second Mating	Third Mating	Fourth Mating
No. 10 Alc.	Alc. male 4, 1 young died in 6 days	Alc. male 4 0	Alc. male 6 0	Alc. male 4, 1 embryo *in utero* 2 weeks after
No. 12 Alc.	Alc. male 5 0	Alc. male 5 0	Alc. male 4 0	.....
No. 11 Alc.	Alc. male 6 0	Alc. male 6 0	Alc. male 5; 2 premat. still-born	Alc. male 4 0
No. 13 Nor.	Alc. male 5 1 still-born	Alc. male 5 0	Alc. male 4 0	.....
No. 17 Nor.	Etherized male 1 0	Etherized male 1 0	.....	.....
No. 18 Nor.	Alc. male 5 0	Alc. male 5 0	Alc. male 6 0	.....
No. 7 Nor.	Etherized male 2; 2 premat. still-born	Etherized male 2 0	Etherized male 2 0	.....
No. 14 Nor.	Etherized male 3 0	Etherized male 3 0	.....	.....
No. 19 Nor.	Alc. male 4 0	Alc. male 6; 1 still-born	Alc. male 6 0	Alc. male 5; 4 small, active, only one-half size, but living
No. 15 Nor.	Alc. male 6; 2 still-born	Alc. male 5 0	Alc. male 5; 2 died fourth week of convulsions	Nor. male; 1 normal vigorous young

The other records are easily understood.

These experiments have suggested many questions still to be solved, some of which are now being tested, such as the length of time necessary to treat an animal before the resulting offspring is affected, whether this time is equally long for both sexes, and what amount of individual variation may exist. An important point to ascertain is whether the effects of the alcohol treatment are permanent, or does the animal recover after a time and again become capable of giving normal offspring. One of the most valuable problems is to regulate the treatment in such a manner as to induce a definite type of defect with a given kind or degree of treatment. The structure or morphology of the monsters and defective

offspring which occur is to be carefully studied. Many other points might readily be suggested.

Definite and well-controlled experiments with alcohol and other substances on the mammalian offspring have not been sufficiently studied. The work is really in its beginning, and while there is much evidence to show that various toxic agents do affect and modify the offspring, facts are badly needed to demonstrate the regularity and manner of this modification. The present experiments seem to me to prove in a convincing way that alcohol may readily affect the offspring through either parent, and that this effect is almost fatal to the existence of the offspring when the parents have been treated with even fairly large doses of alcohol. Many of the cases seem to indicate further, that the tissues of the nervous system of the offspring are particularly sensitive in their responses to the induced conditions.

My assistant, Miss Craig, has aided me greatly throughout almost the entire progress of these experiments. Last year during my absence abroad she assumed entire control of the animals, and I am indebted to her for this efficient assistance.

SUMMARY

Guinea-pigs have been treated with alcohol in order to test the influence of such treatment on their offspring. Male and female animals are given alcohol by an inhalation method until they begin to show signs of intoxication, though they are never completely intoxicated. They are treated for about an hour at the time, six days per week. The treatment in some of the cases has now extended over a period of nineteen months. The animals may be said to be in a state of chronic alcoholism.

Fifty-five matings of the alcoholized animals have been made, forty-two of which have reached full term and are recorded.

From these forty-two matings only seven young animals have survived, and five of them are unusually small, though their parents were large, vigorous guinea-pigs. The following combinations were made:

1. Alcoholic males were mated to normal females. This is the paternal test, and is the really crucial proof of the influence of alcohol on the germ cells, since the defective offspring in this case must be due to the modified spermatozoa, or male germ cells, from which they arise. Twenty-four matings of this type were made, fourteen of which gave no result or very early abortions; five still-born litters were produced, consisting of eight individuals in all, and five living litters containing twelve young. Seven of these twelve died soon after birth, and only five have survived. Four of the survivors are from one litter and the fifth is the only living member of a litter of three.

2. Normal males were mated with alcoholic females. This is the maternal test. In such cases the alcohol may affect the offspring in two ways—by modifying the germ cells of the mother or acting directly on

the developing embryo *in utero*. Only four such matings were tried. One gave no offspring; three living litters were born, one consisting of three premature young that died at birth, while the other two litters consisted each of one young, which have survived. The alcoholic treatment in one of the last cases was only begun after the mother had been pregnant for about three weeks.

3. Alcoholic males were mated to alcoholic females. This is the most severe test, both parents being alcoholic. Fourteen such matings gave in ten cases no offspring, or very early abortions. Three still-born litters were produced, consisting in all of six individuals, while only one living young was born. *This single offspring from the fourteen matings died in convulsions on the sixth day after birth.*

The young that have died in the experiment showed nervous disorders, many having epileptic-like seizures, and all died in convulsions.

Nine control matings in the same group of animals have given nine living litters, consisting in all of seventeen individuals, all of which have survived and are large, vigorous animals for their ages. Two young from non-alcoholic parents died, but this mother also died two days later. Her diseased condition doubtless affected the suckling young.

Forty-two matings of alcoholic guinea-pigs have given only eighteen young born alive, and of these only seven, five of which are runts, survived for more than a few weeks, while nine control matings have given seventeen young, all of which have survived and are normal, vigorous individuals. These facts convincingly demonstrate the detrimental effects of alcohol on the parental germ cells and the developing offspring.

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IS THE CONTROL OF EMBRYONIC DEVELOPMENT A PRACTICAL PROBLEM?

By CHARLES R. STOCKARD.

(*Read April 19, 1912.*)

Under favorable natural conditions two normal parents should, and usually do, produce a vigorous normal offspring. When, however, the conditions of development are modified or if in the second place the parents are not entirely normal the offspring is usually more or less defective. I shall attempt to show that the proper development of the offspring is dependent upon two main factors, first the physical qualities of the parental germ cells, and second the environment in which the embryo develops.

One is at first sight apt to think that deformities and defects are rare among men and other animals; but closer observation will show that the really structurally perfect individual is rather exceptional. Gross anatomical defects or monstrosities are frequently found among all animals, while lesser defects of minor importance are to be observed in a majority of individuals. These defects often cause no inconvenience, and indeed, we may be ignorant of their presence, since they are generally internal. Yet many apparently normal individuals sooner or later suffer or may actually die from some hidden developmental imperfection. The well-known congenital defects of the heart and other parts of the vascular system, digestive tract, etc., as well as the numerous developmental arrests in various parts of the body constantly remind the observer of the great loss in ability and energy that the race suffers as a result of faulty development.

These defects in construction must be considered a disease which causes the death of about 23 per cent. of the human race before or shortly after the time of birth (Sullivan's studies and French statistics), and handicaps a certain proportion of the survivors through-

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out their lives. We carefully study and use all known precautions to protect ourselves against post-natal infections and diseases, and much interest and time is given to combating the causes, yet little is said and scarcely anything done towards a control of development, or the hygienic protection of the developing individual.

This is really a morphological problem and is as truly a part of the fight against disease as is the treatment of abnormal physiological processes. It is not all of morphology to describe and study the detail of bodily structure, but its important task is to understand and analyze that structure, and if possible control and regulate its formation: and thus, if properly developed its goal is to relieve the race of its great structural disease—a disease which affects more individuals than any other one malady of man.

To most persons the above task seems at first thought a futile undertaking, and any one suggesting such control or preventive treatment might be interpreted as indulging in fanciful speculation. Yet the data available from the studies of defective persons in different countries of the world, and the experimental evidence furnished by work on lower animals makes the correction or prevention of developmental defects seem even today a problem to be practically handled to a slight degree at least.

To proceed as with any other disease, we must first ascertain the cause of these conditions, as the possibility of a cure depends upon the nature of the cause.

Are monstrosities and defective development due to some innate change within the germ cells of the parent, thus being incurable, as many former workers would have us believe? Or, are they due to changes produced in the germ cells by the action of some unusual condition in the body of either the male or female parent, or finally may they not be due to an unusual environment acting upon the developing embryo itself? In both of the latter cases the conditions are open to regulation or control. These questions may only be solved experimentally and the experiments have proven that the great majority of monsters are due to the action of unusual conditions upon either the parental germ cells or the developing embryo. There may be some changes of form or variations in animals which are due to

innate changes in the germ-plasm but even these when fully understood may possibly be shown to result indirectly from some change in the chemical surroundings.

First to consider the modifications induced in the developing egg or embryo by a strange chemical environment. It has been found for the eggs of a number of animals that develop normally in sea-water that when certain chemicals are added to their environment they develop into various unusual forms.

I experimented for several years on fish's eggs and found that on adding any one of a large series of salts to the sea-water that the eggs developed abnormally and gave rise to a great number of monstrous individuals. The types of the monstrosities were variable, and the same kind of monster often resulted from different treatments. This was to be expected, but the important problem was to produce some definite type of monster in great numbers with any given treatment. This I finally succeeded in doing and in some experiments got as many as 90 per cent. typical cyclopean or monophthalmic monsters. These types of monsters first occurred in solutions of $MgCl_2$ in sea-water. In such solutions as many as 50 in 100 eggs formed one-eyed cyclopean embryos. Since Mg has the power to inhibit activity in animals and so acts as an anæsthetic I determined to try the action of a number of such substances on the developing eggs to ascertain whether they might also inhibit the lateral migration of eye parts. Alcohol, ether, chloroform, chloreton, etc., were employed and cyclopean monsters resulted from eggs developing in all of these substances. Alcohol gave the most decided effects and inhibited the normal production of eyes in almost all cases. All of these anæsthetics act more particularly upon the central nervous system of the adult and it is important to find that the development of the nervous system is also especially affected by them. In alcohol solutions the embryos showed almost every gross abnormality of the brain which is known to occur, and the spinal cord was often defective.

I have repeated the experiments of Féré with hen's eggs and find that when these eggs are exposed to fumes of alcohol many abnormal chicks result. When hen's eggs are placed in closed dishes over

evaporating 95 per cent. alcohol enough of the fumes penetrate the shell and enter the contents of the egg to cause the developing chick to form abnormally.

McClendon has lately found that an excess of CO_2 and other substances also cause cyclopia and brain abnormalities. Many other workers have shown the effects of the environment on the developing egg.

It is, therefore, proven that the experimenter has the power to take an egg which would normally give rise to a perfect animal and by proper treatment he may cause it to form a typically abnormal individual. The monster may in many cases be able to survive and move about. No one can question that in these experiments the unfavorable environment modifies the form of the resulting individual.

Does this also occur in embryos developing in the mother's body? Children are born which exhibit the same types of deformities as those described above. Syphilitic mothers usually abort or give birth to abnormal children and there is much evidence to indicate that an alcoholic mother is more apt to produce an abnormal child than is a non-alcoholic mother.

Tubal pregnancies are common among women with venereal diseases and in such cases the embryo must necessarily develop under abnormal environment, having a poor surface for placental attachment in a region not adapted to the conditions of pregnancy. The conditions for embryonic nutrition are poor. Mall has found that while only 7 per cent. of uterine pregnancies in his records contained pathological embryos, that 96 per cent. of the embryos in tubal pregnancies were pathological, only 2 in 46 specimens being normal. This is strongly indicative of an abnormal environment as the cause of abnormalities. If these monsters were due to inherent tendencies in the germ cells one should not expect more abnormal tubal than uterine embryos.

Among lower mammals it has been shown that dogs fed on alcohol produce deformed and otherwise defective pups. I am now conducting a series of experiments with guinea pigs which show that a female treated with alcohol during her pregnancy will often

abort or produce defective young, while the control animals are giving birth to normal young. Many more cases could be cited if time permitted.

Experiments on lower animals, therefore, show and human statistics seem to indicate that the cause of structural disease is often an abnormal developmental environment. To prevent such a disease the developmental conditions must be controlled and rendered as nearly normal as possible.

The second consideration is whether abnormal chemical environment may act on the parental germ cells in such a manner as to cause them to change and become incapable of giving rise to a normal individual. It is well known that certain disease toxins such as that of syphilis and substances such as alcohol and lead effect various body tissues so as to render them unfit for normal physiological activity. It is, therefore, only logical to suppose that the same or similar substances may effect the germ cells and so derange their chemical constitutions as to cause them to give rise to offspring of peculiar structure and qualities.

Bertholet has found that alcohol has a particular affinity for the reproductive glands just as it does for the nervous system. In examining the structure of the testicles from a large number of chronic alcoholics it was shown that spermatozoa were absent entirely or degenerate in form (azoospermy) in a majority of the cases. It is doubtless true that the ability of the spermatozoa to accomplish normal fertilization would be affected long before any definite structural change could be observed.

The crucial case is the treatment of the male in such a way as to render his spermatozoa unable to produce a normal development when combined with a healthy egg from a normal female. In this case the action must of necessity be on the germ cell only and not on both the egg and embryo as it might be in treating a female mammal.

It must be recognized that an individual owes its structure and character to the peculiar chemical constitution of the germ cells from which it arises. The germ cells of two species of animals are

probably as different chemically as the animals are morphologically. Therefore, if the chemical nature of the germ cells is disturbed or injured by the action of poisons in the animal's blood they will probably show this injury in the type of individual to which they give rise.

Constantine Paul long ago found in studying 88 cases of pregnancy among women lead workers that 71 resulted in abortion, premature labour, or stillbirth while only 17 children were born alive and of these five died within the first year. Several of these women later produced healthy children after leaving this work. (This indicates that when the cause is known for defective development the cure may often be established by its removal.) Lead not only effects the developing fœtus but also acts directly upon the germ cells as is shown in the case of men working in lead while their wives were not exposed to the poison. Many of the offspring from such fathers are aborted and the children born are epileptic, feeble-minded or generally defective.

To return to the results furnished by the guninea pig experiments referred to above—I have chosen healthy individuals and treated them daily with the fumes of 95 per cent. alcohol to about the point of intoxication. Feeding alcohol and giving it by stomach tube was first tried, both of these methods were unsatisfactory as the guinea pigs did not take alcoholic food in sufficient quantity and the stomach tube disturbed the animals to such a degree that I feared the experimental result might be vitiated even though it could be partially controlled. The inhalation method is perfectly satisfactory; the animals are placed in a copper tank having a screen floor which holds them above the evaporating alcohol. The alcohol is breathed directly into the lungs and affects the animals readily, in much the same manner as weak treatments of ether or chloroform would. The animals are thus put into a condition of chronic alcoholism, being almost intoxicated six times per week. Many of these guinea pigs have been killed and their lungs, liver and other organs examined and found to be perfectly normal so far as their appearance goes. The conjunctiva over the eyes is very often affected by the fumes, during the beginning of the treatment the eyes often

become white, this is transitory in most instances and the eyes finally clear again and remain in a normal condition from then on. Most of the specimens have fattened under the alcohol treatment.

The matings have been made in such a fashion as to test several questions. First, alcoholic males are mated with normal females, paternal influence, the crucial test for the effect upon the germ cells. Second, alcoholic females are paired with normal males, the maternal influence plus the direct action on the developing embryo. Lastly, alcoholic males and females are paired.

The results of 40 such matings are shown in Table I. The decided effects of the alcoholic treatment are seen when the records are compared with those of the normal guinea pigs.

TABLE I.

MATINGS OF ALCOHOLIZED GUINEA-PIGS.

Condition of Animal.	Number of Matings.	No Result or Early Abortion.	Stillborn Litters.	Number of Stillborn.	Living Litters.	Early Deaths.	Surviving Young.
Alc. male × nor. female	24	14	5	8	5	5	5 1 + 4
Nor. male × alc. female.....	2	1	0	0	1	0	1 Preg. 2 in utero, 1 deformed.
Alc. male × alc. female.....	14	10	3	5	1	1 died 6th day	0
Summary.....	40	25	8	13	7	6	6 6 in 25
Nor. male × nor. female. *Control.*	8	0	0	0	8	0	15 15 in 15

In the 24 cases in which normal females were mated with alcoholic males, 14 gave negative results. Some of these probably aborted early as the parents were all fertile and the female is apt to eat the young before they have been observed when they are born prematurely. Five of the matings gave stillborn young, in some cases they were born a little before term. Litters were born alive but the young died soon after showing many nervous symptoms, such

as epileptic-like seizures, and all died in convulsions. Only two litters consisted of normal offspring and these young, five in all, seem healthy though unusually small. It is thus seen that in 24 matings of *normal* females with alcoholic males only two gave normal results. Whereas in the control animals all matings have resulted in the production of normal offspring.

Only two matings were made between normal males and alcoholic females. One of these gave no result or was possibly aborted very early and lost, while the other mating produced one female offspring that lived to become pregnant by an alcoholic male. This last mentioned female was killed by accident, two embryos were found *in utero* one of which was deformed.

Fourteen matings were made between alcoholic males and females. Ten gave no result or aborted very early and were eaten, while four cases showed the following records. One young was born weak and died in convulsions on the sixth day after birth. Two cases of premature births of dead young. One female had young *in utero* when killed.

TABLE II.

SUCCESSIVE MATINGS OF TEN FEMALES.

Animal.	1st Mating.	2d Mating.	3d Mating.	4th Mating.
No. 10 alc.	Alc. male 4 = 1, young died 6th day.	Alc. male 4 = 0.	Alc. male 6 = 0.	Alc. male 4 = 1, embryo in utero 2nd week.
No. 12 alc.	Alc. male 5 = 0.	Alc. male 5 = 0.	Alc. male 4 = 0.	
No. 11 alc.	Alc. male 6 = 0.	Alc. male 6 = 0.	Alc. male 5 = 2, premature, still-born.	Alc. male 4 = 0.
No. 13 nor.	Alc. male 5 = 1, stillborn.	Alc. male 5 = 0.	Alc. male 4 = 0.	
No. 17 nor.	Eth. male 1 = 0.	Eth. male 1 = 0.		
No. 18 nor.	Alc. male 5 = 0.	Alc. male 5 = 0.	Alc. male 6 = 0.	
No. 7 nor.	Eth. male 2 = 2, premature, stillborn.	Eth. male 2 = 0.	Eth. male 2 = 0.	
No. 14 nor.	Eth. male 3 = 0.	Eth. male 3 = 0.		
No. 19 nor.	Alc. male 4 = 0.	Alc. male 6 = 1, stillborn.	Alc. male 6 = 0.	Alc. male 5 = 4, small but active.
No. 15 nor.	Alc. male 6 = 2, stillborn.	Alc. male 5 = 0.	Alc. male 5 = 2, died 4th week.	Nor. male = 1, normal young.

These results stand in marked contrast to the records of the control, which show all normal conceptions and normal offspring.

The second table shows the results of successive matings in ten of the females. The varying success of the conceptions in the same individual are striking.

Nice has quite recently recorded a similar series of experiments with alcohol on mice. Alcohol was given to the mice in their food. Nice finds that while there was a certain fatality among the offspring from alcoholic parents as compared with those from normal parents, where there was no fatality, yet nevertheless the offspring of alcoholic parents actually grew faster than those from the control. This may indicate that alcohol is not equally poisonous in its effects upon all animals, as might really be expected. The germ cells of mice may be more or less immune to the action of alcohol. It is well known that the action of alcohol is different in its effects on individuals from different human families. Some alcoholics show chiefly nervous disorders, hallucinations, delirium, etc., while others may have no nervous symptoms but exhibit various derangements of the digestive glands, kidneys, etc., or may have a fatty degeneration of almost all organs.

Finally it may be concluded that the experimental evidence goes to show that the development of an offspring may be modified by either treating the parents so as to affect their germ cells or by subjecting the developing embryo itself to unusual or injurious conditions.

The causes of many congenital defects are therefore known. It is possible to control embryonic development to such an extent as to produce abnormal structures. May not the proposition be reversed and unfavorable environments be treated in such a manner as to render them favorable to normal development? Diseased mothers may in some cases, at least, be made fit for the function of reproduction.

The regulation of structural disease becomes then a problem of morphology and hygiene. It is most important, and must precede,

or go before, the selective mating of human beings or the eugenics movement. The most intellectual will rarely submit to direction in choosing a mate, yet every productive pair will welcome any possible means of improving the quality of their offspring.

While preventive measures are being used to protect the postnatal life of the individual, why not guard as far as possible its prenatal development?

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An Experimental Study of the Influence of Alcohol on the Germ Cells and the Developing Embryos of Mammals.

By

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The eggs of lower vertebrates which develop outside the maternal body may be induced to give rise to abnormal or monstrous forms when subjected to various unusual conditions. By regulating the chemical environment in which the eggs develop definitely typical defects may be produced in great numbers. The same type of defect may be caused by a variety of substances. This fact was shown by STOCKARD '10 in studies on cyclopia and also recently by MCCLENDON in similar experiments. The occurrence of the same defect under various conditions might be expected and has often been observed by numerous workers.

The important fact, however, is that under certain conditions a particular defect can be made to occur in a large number of the embryos.

In some of the alcohol experiments (STOCKARD '10) on fish's eggs dozens of embryos with typical brain and eye defects occurred while few or no other types of deformities existed. The experimenter has the power in these cases to predict, with at least a limited degree of certainty, the type of deformity which will result from a definite intensity of a certain treatment. Embryonic development in such cases may be partially regulated or controlled.

BARDEEN '07 showed that when normal eggs of the toad are fertilized by spermatozoa which have been modified by treatment

with the Roentgen Rays the eggs develop abnormally. O. HERTWIG '11 has since recorded similar results by treating the spermatozoa of frogs with radium. These experiments show convincingly the influence of the male germ cell on the development and structure of the embryo. Thus in the lower vertebrates it has been possible to modify development by treating either the egg or spermatozoon.

The question now arises whether the germ cells and developing embryos of mammals may be similarly affected. The conditions in mammals are more complex since the embryo develops internally, and the substances administered to the body of the parent may induce secondary effects which will modify or confuse the results. Should a substance be secured that will act directly upon the germ cells or the tissues of the developing embryo within the parental body, then one might expect to regulate the action of this substance in much the same manner as in experimenting upon eggs developing in sea-water. From a number of recent observations it would seem that alcohol is just such a substance as is required.

It is an accepted fact that alcohol may cause changes and degeneration in many of the body tissues. How then can the reproductive tissues escape, or may they be affected without seriously injuring other tissues? BERTHOLET, 1909, made an extensive examination of the influences of alcohol upon the histological structure of the reproductive glands and found much degeneration and atrophy in the testicular parenchyma of chronic alcoholics, both young and old. NICLOUX has shown that alcohol has a marked affinity for the reproductive glands and that alcohol may occur, as such, in the testicular tissues and in the seminal fluid of mammals. The ripe spermatozoa may, therefore, be bathed in a weak solution of alcohol shortly before fertilizing the egg, and if affected by the alcohol the spermatozoon may cause the developing eggs to give rise to a defective or deformed individual.

NICLOUX has also demonstrated the passage of alcohol from the blood of the mammalian mother into the tissues of the embryo (guinea-pigs and dogs). After a short period of time the amount of alcohol in the blood of the foetus is about equal to that in the blood of the mother, while there is really more alcohol in a given weight of foetal tissue than is to be found in an equal weight of liver tissue from the mother. The reality of the passage of alcohol from the mother to the foetus demonstrates the possibility of the intoxication of the foetus.

The experiments on mammals do not then really differ greatly from those on the lower vertebrates, where the externally developing eggs may be placed directly in various unusual solutions; since the egg or embryo, although within the mother's body, is readily bathed or impregnated by the alcohol contained within the mother's blood.

The few experiments upon the influence of alcohol on the mammalian offspring are not at all conclusive and are somewhat contradictory.

COMBEMALE and HODGE studied the effects of alcohol upon the offspring of dogs and recorded injurious effects, though their experiments were performed on very limited numbers of individuals.

Quite recently NICE has recorded experiments which seem to show that alcohol exerts a very slight effect upon the offspring of mice. He has given small doses of alcohol mixed with the food and also supplied 35 % alcohol instead of water for the animals to drink. Much of the alcohol probably evaporated from the food before it was eaten. Animals treated in this way gained in weight over the control, and their offspring excelled the control in rate of growth. The fecundity of the treated mice was greater than that of the control but there was also a greater mortality of the offspring from the treated parents. None of the control young died while 11.1 % of the alcoholic young died soon after birth. There were no abortions, no still-births and none of the young were deformed.

Mice may possibly be peculiarly resistant to alcohol though we should rather think that they received too little to give a pronounced effect, yet it was sufficient to cause a certain fatality among the offspring.

The few studies on mammals have failed to produce convincing evidence of the specific actions of alcohol. Yet the statistical data from observations on defective human beings would indicate that alcohol had a special affinity for the developing nervous system [1]). The experiments upon the influence of alcohol on the developing fish embryo (STOCKARD '10) demonstrated that alcohol did have a specific affinity for the central nervous system and caused the brains of these embryos to exhibit numerous deformities while the organs of special sense were also affected.

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[1]) The senior author has given a somewhat extensive review of the literature pertaining to this subject in the Archives of Internal Medicine, 1912.

Method and Material.

The experiments here recorded were undertaken in order to ascertain whether alcohol did exert a marked influence on the germ cells and developing embryos of mammals, and if possible to demonstrate the nature and mode of action of this influence. Alcohol was employed as an agent since it may be given to guinea-pigs without greatly disturbing their normal physiological processes and so does not produce marked conditions which would secondarily effect the results. As before mentioned alcohol may remain as such in the blood and tissues of a mammal and may thus act directly upon the embryo just as it would when added to the sea-water in which fish's eggs were developing. It is an active substance, and, therefore, lends itself admirably to experimental use.

The experiments have been conducted on guinea-pigs since they breed fairly rapidly and rear their young without much difficulty in the laboratory. Strong healthy stock has been chosen and the animals have been carefully handled. All have remained in vigorous health and most of them have increased in size and fattened during the progress of the experiment. The males and females have been kept separated and individual pairs were mated from time to time.

The animals are first tested by normal matings and found to produce normal offspring. The alcoholic treatment is then begun on a given number of individuals and the males and females mated in different combinations according to whether they are alcoholics or normal. An alcoholic male is mated with a normal female, the paternal test, this is the crucial test of the influence upon the germ cells as here the defective offspring must be due to the chemically modified spermatozoon from which it arises, since the egg and the mother in which the embryo develops are both normal.

Normal untreated males are paired with alcoholic females, maternal test, in this case the defective offspring may be due either to a modified ovum or to the fact that it developed in a mother with alcoholic blood, therefore supplying an unfavorable developmental environment. Lastly, its condition may be due to both of these causes. The mammalian mother has two chances to injure an offspring, either by producing a defective egg or secondly by supplying an unfavorable or diseased environment in which the embryo develops.

The final combination is the mating of alcoholic individuals, this of course offers the greatest chance for defective offspring.

Alcohol is administered to the animals by inhalation. It was first given with the food, but the animals did not relish it, and therefore took less food. It was then given by stomach tube but this method so upset the animals that the results might have been modified by their poor bodily conditions and the bad state of their stomachs. The inhalation method is entirely satisfactory, the guinea-pigs thrive and usually gain in weight during the experiment, they have good appetites and are in all respects apparently normal. The only indication of the effects of the treatment is shown by the quality of offspring they produce.

The apparatus used for giving the alcohol consists of an air tight copper tank 36 inches long by 18 inches wide and 12 inches deep with a sloping bottom draining to the center. Over this bottom is placed a wire screen and below the screen cotton soaked with 95% alcohol is spread. The tank is closed and allowed to stand until the atmosphere within is saturated with alcoholic fumes. A ventilation system is so arranged that a given quantity of alcohol fumes may be driven through the tank in a given time, but it has not seemed advisable to use this device as the degree of intoxication is a better index to the physiological response of the animals. Their resistance to the fumes is changeable. The guinea-pigs, three or four at the time, are placed on the wire screen above the evaporating alcohol, the tank is again closed and the animals are allowed to remain until they begin to show signs of intoxication, though they are never completely intoxicated. They usually inhale the fumes for about one hour. The animals are treated in this way for six days per week and some have now been treated over a period of about nineteen months. None of the effects are due to want of air since the same number of guinea-pigs may remain for hours in this closed tank without showing any signs of discomfort when there are no fumes present.

In order to avoid handling the females during late pregnancy a special treating cage is devised for them. An ordinary box run with a covered nest in which the animal lives is connected by a drop door with a metal lined tank having a similar screen arrangement to that described for the general treatment tank. The pregnant animal may be driven daily into the tank and thus treated with alcohol fumes throughout her pregnancy without having to be handled or moved about in any way that would tend to disturb the developing foetus.

Results.

During the vapor treatment the animals usually react in a manner quite similar to their behavior in weak fumes of ether or chloroform. The majority of them sit quite motionless and sniff their noses for a time and then become somewhat drowsy. A few individuals, however, are excited by the treatment and run about the tank becoming sexually excited and may often fight other animals savagely. One of the males fights and bites so vigorously while taking the fumes that he has to be treated separately from all others. The fumes thus have a different influence upon the behavior of different individuals in much the same way that alcoholic intoxication expresses itself differently on different human beings.

During the first few weeks of the treatment the fumes cause the eyes to water so that tears run over the face. The nose and mouth also become moist and the animals sniff almost constantly. Alcoholic fumes are very irritating to the mucous membranes at first. The conjunctiva of the eye becomes irritated and finally opaque in some instances, so that the eye takes on a white appearance. The tissues seem, however, to develop a resistance to the fumes. The eyes become clear after a few months and never again become opaque. The nasal mucosa also ceases to secrete excessively unless the animal is left in the tank for an unusually long time.

Many of the guinea-pigs have been killed after treatments of different duration up to fifteen months and all of their viscera carefully examined and the reproductive glands studied microscopically. In no case have we found any changed structures due to the alcoholic treatment. The lungs, liver, stomach, intestines, kidneys, reproductive glands, brain and all other parts appear perfectly normal. The general health and behavior of the animals also indicate that they are in good condition. As before mentioned several animals have been partially castrated during the experiment. One of the reproductive glands was removed and examined microscopically. In all cases the germ cells, ova or spermatozoa, were found to exhibit perfectly normal structure. One cannot claim, therefore, that this treatment is excessively severe or greater in proportional amount than the alcohol a human being often takes. The matter of fact is that these animals have never been completely intoxicated but receive only enough alcohol six times per week to affect their nervous states.

They may be compared to a toper who drinks daily but never becomes really drunk.

While the bodies of these animals show no direct effects of the alcohol, the conditions of the offspring to which they give rise exhibit most strikingly the effects of the alcoholic treatment. The results of mating the alcoholized guinea-pigs are summarized in Table I.

Table I.

Effects of Alcohol on the Offspring of Guinea-Pigs.

Condition of animal	No. of matings	No result or early abortion	Still-born litters	No. still-born young	Living litters	Young dying soon after birth	Surviving young
Alcoholic male by normal female .	24	14	5	8	5	7	5[1]
Normal male by alcoholic female	4	1	0	0	3	3 premat.	2[2]
Alcoholic male by alcoholic female	14	10	3	6	1	1 6th day	0
Summary	42	25	8	14	9	11	7[3]
Normal male by normal female . Control	9[4]	0	0	0	9	0	17

Fifty-five matings of treated animals have been made. Forty-two of these have now reached full term and are recorded. Thirteen matings are not yet due. From the forty-two matings only seven young survived, and six of these are still living, five of which are small for their ages though their parents were unusually large strong animals.

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[1]) Four survivors in one litter, and one was a member of a litter of three, the other two died immediately after birth.

[2]) One lived to become pregnant with two young in utero, one deformed. Other survivor normal, the mother was not treated until after first two or three weeks of pregnancy.

[3]) Of thirty-two young born only seven have survived.

[4]) One other non-alcoholic mating was made from which two young resulted, they died after the second and fourth days respectively and the mother died two days later, her diseased condition no doubt affected the suckling young. They have for this reason not been included in the normal control.

The conditions of the animals in the mating pairs are shown in the first column of the table and the total results of the mating are indicated in the following columns.

The first horizontal line gives the records when alcoholic males are paired with normal females. Twenty-four such matings were made. Fourteen of these gave negative results or resulted in early abortions. Many embryos were aborted during very young stages and some of these were deformed, though they were generally in such poor conditions after being cast out into the cages that little could be learned from them. They were partially or completely eaten by the mother in most cases. The males were always kept for a number of days with the females during favorable periods and conception should have occurred in all cases, as it did in the control matings. Only ten of the twenty-four matings resulted in conceptions which ran the full term. Half of these, or five, were still-born litters. There were three still-born litters of two young each and two of one individual each. Most of these were slightly premature, their eyes being closed and the hair sparce on their bodies. (A normal guinea-pig at birth is well covered with a hairy coat, its eyes are open and it very quickly begins to run about actively.)

Five litters of living young were born. One litter consisted of only one young, a weak individual that grew very little and died after six weeks. Two litters contained two young each. The members of one of these litters died during the first and fourth weeks having been weak and small since birth. Both of those in the other litter were in a similarly feeble condition and died before the first month. One litter contained three young, two of these died immediately after birth; the other one is still alive though small for its age. The fifth litter contained four young all of which are runty though their parents were unusually large animals. Thus out of twenty full-term young of which only twelve were born alive but five individuals have survived and these are unusually small and very shy and excitable animals.

It is a point of some interest that all of the young animals that died showed various nervous disturbances having epileptic-like seizures and in every case died in a state of convulsion.

The important fact in the above cases is that only the father was alcoholic, the mother being a normal vigorous animal. This experiment clearly demonstrates that the paternal germ cells of mammals may be modified by chemical treatment

to such a degree that the male will beget abnormal offspring even though he mate with a vigorous female. A reconsideration of the figures in the first line of the table shows really how decidedly the injured spermatozoon expresses itself in the fate of the egg with which it combines.

The second line of the table shows the results of matings between alcoholized females and normal males. These matings might be expected to give more marked results than the previous ones, since in the treated females not only the germ cells may be affected but the developing embryo itself may be injured by the presence of alcohol in the blood of the mother. NICLOUX has shown that alcohol may pass directly from the maternal blood into the embryonic tissues of a guinea-pig.

The spermatozoon, however, is probably a more sensitive structure than the egg and is easily injured or killed by slightly abnormal conditions. It might possible be that when such specialized cells swam for even a short time in seminal fluid containing a trace of alcohol that their chemical nature would be so decidedly disturbed as to render them incapable of inducing normal development after impregnating the eggs. At any rate the few cases at present available seem to indicate that the effect on the offspring is equally as great when it is produced by an alcoholic father as by an alcoholic mother.

There are only four matings between alcoholized females and normal males. One of these gave a negative result or was possibly aborted very early. Three living litters were born. One litter consisted of three premature young which died shortly after birth. The remaining two litters each contained only one young but these two animals survived. One of these guinea-pigs was born after the mother had been treated for three and one half months. The offspring was weak and small for several months after birth but finally recovered and developed into a normal animal. This animal, a female, was mated with an alcoholic male and became pregnant. Unfortunately she was killed by accident and on examination her uterus was found to contain two embryos of 33 and 32 mm. in length. One of these embryos was deformed and showed very decidedly degenerate and feebly developed hind legs. The posterior end of its body was also poorly formed. This is of interest since all of the affected offspring of alcoholic guinea-pigs are weak in their hind extremities and drag their legs. Yet none were so modified as to show a noticeable

structural defect except this embryo, which had one alcoholic grandmother and an alcoholic father.

The only other survivor from an alcoholic mother is strong and full grown for its age. The mother had been treated for only one and one-half months when the offspring was born so that she was normal during the first two or three weeks of pregnancy. No doubt the early stages of development are more easily modified to produce significant defects than are the later. This question is being more fully tested on guinea-pigs with experiments now in progress. STOCKARD has shown, however, in treating fish's eggs that the period at which the treatment is applied is a most important factor in determining the type of defect or modification which will result. Certain salts, different strengths of magnesium chloride for example, which give pronounced effects when added to the sea-water containing eggs in early developmental stages may really be ineffective after the eggs have developed beyond these stages. In the case under consideration the offspring might not have fared so well if the alcoholic treatment had been started on the mother a few weeks before conception, instead of three weeks after her pregnancy had begun. This with other points shall be more completely analyzed in future communications on these experiments.

The four matings of alcoholic females and normal males resulted in three living litters consisting in all of five individuals. Three of the young were premature and died shortly after birth while two young survived.

Finally, we may consider the results of pairing two alcoholized individuals. The third line of the table summarizes these data. As might have been anticipated this type of mating has given the highest fatality of all.

Ten out of a total of fourteen matings have given no offspring or early abortions which were in many cases eaten by the mother. Three still-born litters have been produced each consisting of two young. Only one living litter was born from the fourteen matings in which both parents were alcoholic and this litter consisted of but one weak individual which died in convulsions on the sixth day after birth. This is indeed a decided effect of alcohol upon the offspring when one considers the nine control matings, all of which gave living litters containing a total of seventeen individuals all surviving.

Two young which were included in the control and died, should

not really be counted. They died four days after birth and the mother died two days later in a diseased condition. No doubt the poor state of the mother had much to do with the fate of the suckling young. She was an animal that had only been in the experiment for a short time (this was her first mating) and is one of the very few that have contracted disease or died during the nineteen months of the work.

The fourth line of Table I gives a summary of the experiments. There have been forty-two full-term matings, twenty-five of which gave no offspring or early abortions, eight still-born litters have occurred consisting of fourteen individuals, only nine living litters have been born, 21% of the matings, these contained eighteen young and but seven of this number have survived and five of these survivors are runts or small for their ages.

The bottom line of the table shows nine control matings, all have given living litters containing a total of seventeen young, all of them surviving. Two young that died, as stated above, were from a dying mother and are not included in the control.

Records of successive matings of ten of the female guinea-pigs are shown in Table II. The varying ways in which the same individual has responded in different matings is noticeable. Number 10, an alcoholic female, first mated with an alcoholic male gave one young which died on the sixth day after birth. On being remated with the same male No. 10 gave no offspring. Then mated with another alcoholic male gave no offspring. She mated again after several months with the first male and on being killed was found to contain one embryo in utero about two weeks old.

Female No. 15, a normal guinea-pig, shows an instructive record. She was mated with an alcoholic male and gave birth to two still-born young. Mated to another alcoholic male and gave negative result. Remated with the second male and gave two young both of which died of convulsions in four weeks after birth. She was then mated with a normal male as a control and gave one vigorous normal offspring which survived.

The other records are easily understood.

These experiments have suggested many questions still to be solved, some of which are now being tested. The length of time necessary to treat an animal before the resulting offspring is affected, whether this time is equally long for both sexes, and what amount of individual variation may exist. An important point to ascertain

Table II.

Successive Matings of 10 Females.

Animal	1st Mating	2nd Mating	3rd Mating	4th Mating
No. 10 Alc.	Alc. male 4, 1 young died in 6 days	Alc. male 4 0	Alc. male 6 0	Alc. male 4, 1 embryo in utero, 2 weeks after
No. 12 Alc.	Alc. male 5 0	Alc. male 5 0	Alc. male 4 0	
No. 11 Alc.	Alc. male 6 0	Alc. male 6 0	Alc. male 5 2 premat. still-born	Alc. male 4 0
No. 13 Nor.	Alc. male 5 1 still-born	Alc. male 5 0	Alc. male 4 0	
No. 17 Nor.	Etherized male 1 0	Etherized male 1 0		
No. 18 Nor.	Alc. male 5 0	Alc. male 5 0	Alc. male 6 0	
No. 7 Nor.	Etherized male 2 2 premat. still-born	Etherized male 2 0	Etherized male 2 0	
No. 14 Nor.	Etherized male 3 0	Etherized male 3 0		
No. 19 Nor.	Alc. male 4 0	Alc. male 6 1 still-born	Alc. male 6 0	Alc. male 5 4 small, active only $1/2$ size, but living
No. 15 Nor.	Alc. male 6 2 still-born	Alc. male 5 0	Alc. male 5 2 died 4th wk. convulsions	Nor. male 1 normal vigorous young

is whether the effects of the alcoholic treatment are permanent, or does the animal recover after a time and again become capable of giving normal offspring. One of the most valuable problems is to regulate the treatment in such a manner as to induce a definite type of defect with a given kind or degree of treatment. The structure or morphology of the monsters and defective offspring which occur is to be carefully studied. Many other points might readily be suggested.

Definite and well controlled experiments with alcohol and other substances on the mammalian offspring have not been sufficiently studied. The work is really in its beginning, and while there is much evidence to show that various toxic agents do effect and modify the offspring, facts are badly needed to demonstrate the regularity and manner of this modification. The present experiments seem to us to demonstrate in a convincing way that alcohol may readily effect the offspring through either parent, and that this effect is almost fatal to the existance of the offspring when the parents have been treated with even fairly large doses of alcohol. Many of the cases seem to indicate further, that the tissues of the nervous system in the offspring are particularly sensitive in their responses to the induced conditions.

Summary.

Guinea-pigs have been treated with alcohol in order to test the influence of such treatment on their offspring. Male and female animals are given alcohol by an inhalation method until they begin to show signs of intoxication, though they are never completely intoxicated. They are treated for about one hour at the time, six days per week. The treatment in some of the cases has now extended over a period of nineteen months. The animals may be said to be in a state of chronic alcoholism.

Fifty-five matings of the alcoholized animals have been made, forty-two of which have reached full term and are recorded.

From these forty-two matings only seven young animals have survived, and five of them are unusually small though their parents are large vigorous guinea-pigs.

The following combinations were made:

1) Alcoholic males were mated to normal females. This is the paternal test, and is the really crucial proof of the influence of alcohol on the germ cells, since the defective offspring in this case must be due to the modified spermatozoa, or male germ cells, from which they arose. Twenty-four matings of this type were made, fourteen of which gave no offspring or very early abortions; five still-born litters were produced consisting of eight individuals in all, and five living litters containing twelve young. Seven of these twelve died soon after birth and only five have survived. Four of the survivors are from one litter and the fifth is the only living member of a litter of three.

2) Normal males were mated to alcoholic females. This is the maternal test, in such cases the alcohol may affect the offspring in two ways, by modifying the germ cells of the mother or by acting upon the developing embryo in utero. Only four such matings were tried. One gave no offspring, three living litters were born, one consisting of three premature young that died at birth, while the other two consisted each of one young which has survived. The alcoholic treatment in one of the last cases was only begun after the mother had been pregnant for about three weeks.

3) Alcoholic males were mated to alcoholic females. This is the most severe test both parents being alcoholic. Fourteen such matings gave in ten cases no offspring or very early abortions. Three stillborn litters were produced consisting in all of six individuals, while only one living young was born. This single offspring from the fourteen matings died in convulsions on the sixth day after birth.

The young that have died in the experiment showed nervous disorders many having epileptic-like seizures and all died in convulsions.

Nine control matings of the same group of animals have given nine living litters consisting in all of seventeen individuals, all of which have survived and are large vigorous specimens for their ages.

Fourty-two matings of alcoholic guinea-pigs have given only eighteen young born alive and of these only seven, five of which are runts, survived for more than a few weeks, while nine control matings gave seventeen young all of which have survived and are normal vigorous individuals. These facts convincingly demonstrate the detrimental effects of alcohol upon the parental germ cells and the developing offspring.

Zusammenfassung.

Meerschweinchen wurden mit Alkohol behandelt, um den Einfluß einer solchen Behandlung auf ihre Nachkommenschaft zu erweisen. Männliche und weibliche Tiere bekommen Alkohol mittels einer Inhalationsmethode, bis sie Zeichen von Intoxication aufweisen, doch werden sie niemals völlig betrunken gemacht. Sie werden jedesmal etwa 1 Stunde lang an 6 Tagen der Woche behandelt. Die bisherige Behandlung erstreckt sich in einigen der Fälle auf eine Dauer von 19 Monaten. Die Tiere befinden sich sozusagen in einem Zustande von chronischem Alkoholismus.

Es wurden 55 Paarungen der alkoholisierten Tiere vorgenommen, von denen 42 bis zum vollen Schwangerschaftsablauf kamen und hier benutzt sind. Von diesen 42 Paarungen sind nur 7 junge Tiere am Leben geblieben, und 5 von diesen sind ungewöhnlich klein, obgleich ihre Eltern große, kräftige Meerschweinchen sind.

Die folgenden Kombinationen wurden versucht:

1) Alkoholische Männchen wurden mit normalen Weibchen gepaart. Das ist also die Prüfung des väterlichen Einflusses und stellt den entscheidenden Versuch betreffs des Einflusses des Alkohols auf die Keimzellen dar, da in diesem Falle die beeinflußten Spermatozoen oder männlichen Keimzellen die Ursache der defekten Nachkommenschaft sein müssen, von denen sie ihren Ausgang nimmt. 24 Paarungen dieses Typus wurden veranstaltet, von denen 14 überhaupt keine Nachkommenschaft oder sehr frühzeitige Aborte ergaben. 5 totgeborene Sätze enthielten alles in allem 8 Individuen und 5 lebende Sätze ergaben 12 Junge. 7 von diesen 12 starben bald nach der Geburt und nur 5 sind am Leben geblieben. 4 der Überlebenden stammen aus einem Wurf und das 5. ist das einzig Überlebende aus einem Wurf von 3 Individuen.

2) Normale Männchen wurden mit alkoholischen Weibchen gepaart. Das stellt also die Untersuchung des Einflusses der Mutter dar. In solchen Fällen kann der Alkohol die Nachkommenschaft auf zweierlei Weise affizieren: durch eine Einwirkung auf die mütterlichen Keimzellen oder eine Einwirkung auf den sich im Uterus entwickelnden Embryo. Nur 4 solche Paarungen gelangten zur Untersuchung: 1 ergab keine Nachkommenschaft, lebende Sätze wurden 3 geworfen, von denen einer aus 3 frühgeborenen Jungen bestand, welche bei der Geburt starben. Die andern Sätze bestanden aus je 1 Jungen, das leben blieb. Die Alkoholbehandlung in einem dieser letzten Fälle setzte erst ein, als die Mutter bereits seit ungefähr 3 Wochen trächtig war.

3) Alkoholische Männchen wurden mit alkoholischen Weibchen gepaart. Das ist der stärkste Versuch, da ja beide Eltern alkoholisch sind. 14 solche Paarungen ergaben in 10 Fällen keine Nachkommenschaft oder sehr frühzeitige Aborte. 3 tote Würfe wurden hervorgebracht mit im ganzen 6 Individuen, und nur ein einziges lebendes Junge geboren. Dieser einzige Nachkomme von den 14 Paarungen starb in Konvulsionen am 6. Tage nach der Geburt.

Die während der Versuche gestorbenen Jungen zeigten nervöse Störungen, manche hatten epilepsieähnliche Zufälle und alle starben in Krämpfen.

9 Kontrollpaarungen von derselben Tiergruppe ergaben 7 lebende Würfe, die im ganzen aus 17 Individuen bestanden, die sämtlich am Leben blieben und für ihr Alter kräftige Exemplare darstellen.

42 Paarungen alkoholisierter Meerschweinchen haben nur 18 lebende Junge ergeben, und von diesen lebten nur 7, darunter 5 Kümmerlinge, länger als einige Wochen, während 9 Kontrollpaarungen 17 Junge ergaben, welche alle am Leben blieben und normale, kraftvolle Individuen sind. Diese Tatsachen demonstrieren überzeugend den schädigenden Einfluß des Alkohols auf die elterlichen Keimzellen und die sich entwickelnde Nachkommenschaft.

(Übersetzt von **W. Gebhardt.**)

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Fütterungsversuche an Amphibienlarven.

Von **J. F. Gudernatsch** (New York).

(Vorläufige Mitteilung.)

(Der Redaktion zugegangen am 9. Mai 1912.)

Es wurde versucht, der zurzeit im Mittelpunkt des Interesses aller Biologen stehenden Frage, welche Rolle die meisten drüsigen Organe, namentlich die Drüsen mit sogenannter „innerer Sekretion", im Haushalte des Organismus spielen, auf dem Wege der Verfütterung experimentell näher zu treten. Zu den Versuchen wurden im Jahre 1911 Quappen von Rana temporaria und esculenta verwendet, jetzt werden diese Versuche an den gleichen Tieren und an Bufo- und Tritonlarven fortgesetzt. Zur Verfütterung werden Säugetierorgane verschiedentlichen Ursprungs verwendet, vom Pferd, Rind, Schwein, Hund, Katze, Kaninchen usw. Es hat sich bis jetzt gezeigt, daß die Spezies herkunft der Organe ohne wesentliche Bedeutung für ihre Wirkungsweise ist. Verfüttert werden: Thyreoidea, Thymus, Nebenniere, Hypophyse, Hoden, Ovarium, Milz, Leber, Pankreas und Muskel. Die genannten Substanzen werden frisch kurz nach Entnahme aus dem Organismus oder, nachdem sie höchstens zwei Tage auf Eis gelegen sind, verfüttert. Im Jahre 1910 ausgeführte Versuche, den Einfluß von Extrakten obiger Substanzen und solcher verschiedener benigner und maligner Tumoren auf die Entwicklung von Fisch- und Amphibieneiern zu studieren, führten aus äußeren Gründen zu keinen befriedigenden Resultaten.

War es a priori zweifelhaft, ob von Säugern entnommene Organe bei Verfütterung an Amphibien auf deren Entwicklung einen spezifischen Einfluß ausüben könnten, so hat der Verlauf der Experimente alle Zweifel darüber verscheucht. Natürlich bleibt immer noch die Frage offen, ob diese Organe, nachdem sie den Amphibiendarm passiert haben, auf den betreffenden Organismus den gleichen Einfluß ausüben, der ihnen in ihrem Ursprungsorganismus zukommt.

Sehr deutliche Resultate ergeben die Verfütterung von Thyreoidea und Thymus, zweier Organe, die ja auch bei Wachstum und Differenzierung des sich entwickelnden Organismus eine große Rolle spielen. Werden sie verfüttert, so sind beide Organe in ihrer Wirkung auf jene Vorgänge gerade entgegengesetzt und auch der Zeitpunkt, auf dem ihr Einfluß am stärksten in die Erscheinung tritt, dürfte ein verschiedener sein. Die Thyreoidea scheint am raschesten zu wirken, je älter die Tiere bei Beginn der Fütterung sind, bei der Thymus scheint das Umgekehrte der Fall zu sein.

Wird Thyreoidea auf irgend einem Stadium gefüttert, so hört jedes Weiterwachstum der Quappen auf und die Tiere schicken sich sofort zur Metamorphose an. So konnten Quappen, die noch keine Extremitäten besaßen, innerhalb 7 Tagen dazu gebracht werden, Hinter- und Vorderbeine zu entwickeln und den Schwanz zu reduzieren. In diesem Frühjahr ist es gelungen, Quappen, die erst 16 Tage alt (16 Tage nach dem Verlassen des Eies) waren, zur Bildung der Vorderextremitäten zu bringen. Da die Thyreoideafütterung jedes Weiterwachstum unterdrückt, so sind das Resultat derselben ganz kleine (Zwerg-) Frösche. Dabei ist es ganz gleichgültig, auf welcher Altersstufe die Behandlung beginnt oder welche Nahrung vorher verfüttert wurde. Waren die Quappen zu Beginn der Fütterung sehr klein, so sind es die metamorphosierenden Tiere auch, größere Quappen aber ergeben größere Frösche.

Bei der Verfütterung von Thymus sind die Resultate gerade entgegengesetzt. Die Tiere wachsen anfangs sehr rasch, es werden große Kaulquappen erzeugt, je länger dieselben aber unter dem Einflusse der Thymus stehen, um so mehr wird die Differenzierung hinausgeschoben und die Metamorphose eventuell ganz unterdrückt. So kommt es, daß aus demselben Satz die mit Thyreoidea gefütterten Tiere sehr rasch, innerhalb 1 bis 2 Wochen, zur Metamorphose gebracht werden können, während die mit Thymus gefütterten Tiere selbst viele Wochen später, nachdem die Kontrolltiere schon längst metamorphosiert haben, zum größten Teil noch ganz undifferenzierte Quappen sind ohne Extremitäten und teilweise gar nicht zur Metamorphose kommen.

Interessant sind auch Färbungsunterschiede der verschieden behandelten Tiere, z. B. die tiefdunkle Farbe der Thymusquappen (Ausbreitung der Pigmentzellen), die auffallend helle Farbe der Nebennierequappen (Kontraktion der Pigmentzellen) usw.

Ein eingehender Bericht über die Gesamtversuche von 1911 und 1912 sowie über die histologischen Ergebnisse und die Beeinflussung der Regeneration durch die verschiedenen Substanzen wird später gegeben werden.

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Feeding Experiments on Tadpoles.

I. The influence of specific organs given as food on growth and differentiation.

A contribution to the knowledge of organs with internal secretion.

By

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With plate IX.

Eingegangen am 11. Juli 1912.

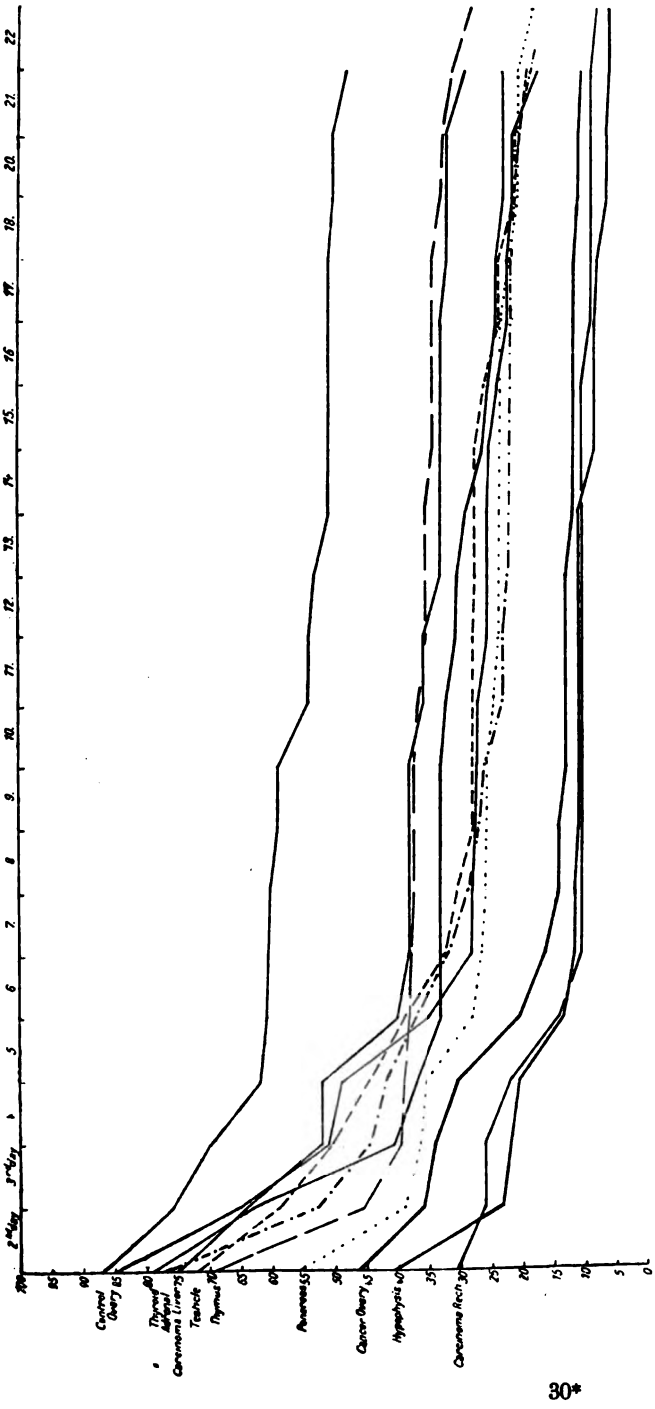
During a stay in the Zoological Station of Naples, spring 1910, an attempt was made to study the influence of various organic extracts on the development of fish (*Belone*, *Gobius* etc.) and amphibian (*Rana*) eggs. The substances used were extracts from mammalian tissues, viz. thyroid, thymus, testicle, ovary, hypophysis, adrenal, pancreas, cancer of ovary, carcinoma of liver and carcinoma of rectum. Different quantities of the extracts, in corresponding degrees of concentration, were added to the sea-water, containing the recently fertilized eggs. The eggs were kept for various lengths of time — up to 20 hours — in this mixture and afterwards transferred into pure sea-water. In every case an influence upon the developing eggs was noticeable; and the disturbances of the normal development caused by the various substances were different. The difficulties met in these experiments were unusually great, partly on account of the rapid decomposition of the extracts brought from New

[1]) My best thanks are due to Prof. KOHN for permitting me to work in his laboratory, furnishing ample material and giving me out of his enormous experience valuable help and advice as well as for carefully revising the manuscript.

York — on board ship they were kept in cold storage, but there was no ice-box in the laboratory — and partly because in that season it was very difficult to get sufficient material; in two months *Belone* eggs were brought to the laboratory only twice. In spite of these inconveniences a great number of eggs were kept under observation up to the time of hatching. Yet the work could not be carried on systematically with sufficient repetitions and control experiments. Besides it was not to be expected that the influences of the various organs on development would allow of any conclusions as to the function of the respective organs, viz. thyroid gland etc. It was more likely that the disturbances of the normal development were of a general type caused perhaps by the change in the osmotic pressure of the surrounding medium etc.

To show that the influences of the various substances upon development were actually different, the following table may be given. It cannot, of course, be used for any generalizations; for it is the result of only one experiment on *Belone* eggs. The curves show the respective percentages of the living and developing eggs after a certain number of days; viz. of each 100 eggs there were on the second day still living: in the control 87, after addition of ovary extract 85, etc. to .... carcinoma recti extract 30; at the beginning of the third day 76, 65, ... 26, etc. After the first day (each 100) a different decline of the curves is already visible. A certain percentage of the eggs, 13 of the control, died, since naturally not all eggs were equally able to develop, others were not fertilized etc. For this reason it may be that on the second day the curves rather run parallel. A striking decline of all curves with the exception of the control and thymus curves appears on the fifth day. On this day in normal development, the heart begins to beat. Many eggs which up to that time remained alive, although probably with diminished energy, seem not to have been able to survive this critical point, the starting of pulsation. From the sixth day on the curves run more or less parallel. The control shows after this time a comparatively strong decline. An explanation for this phenomenon may be that under the influence of the extracts the more feeble eggs were killed in the earlier stages of development, while in pure sea-water they were able to go on developing for some time before their vitality was exhausted.

Table I.



Later on the advice of Prof. KOHN, the various organs have been applied as fresh feeding material. The tadpoles of *Rana temporaria* and *Rana esculenta* were chosen for the experiments. These feeding experiments carried on during the summer of 1911 in the histological laboratory in Prague gave some very interesting results.

The tadpoles were kept in bowls, each containing 15 to 20 individuals, and were fed three or four times a week on the different organs. *Rana temporaria* was used in two sets of different ages. As food were tried: thyroid, liver, adrenal, hypophysis, and muscle from horse, thymus from calf, testicle and ovary from dog or cat. Some organs from rabbits and pigs also were given. The origin of the organs used apparently made no difference in their action. The food was put into the water and was ravenously taken by the animals. With each experiment one group was left unfed as control to test how much nourishment the animals could take from the tap-water which in Prague is very rich on organisms. The water was changed daily, on hot days sometimes twice. This frequent change of water as well as the accumulation of the products of metabolism in the water between the changes may have exerted some influence on the development, yet the prevailing conditions were the same for all animals. Unfortunately it was found impossible to carry a constant current of air or water through the great number of dishes. It was also found impossible on account of the artificial feeding to keep plants in the dishes. The feeding was continued till shortly after the appearance of the fore legs, then the animals cease to take food for some time on account of the transformation of the jaws, and since breathing through the lungs begins the metamorphosed animals leave the water and look for fresh food.

Experiment I.

Rana temporaria in two sets of different sizes. Fig. 1 *a*—*e*, 2 *a*—*e*. Original size on May 23rd 1911 of set I 1.1—1.7 cm, of set II 1.8—2.3 cm.

The differences in size of the animals of the same set diminish somewhat with continued feeding, so that the deviations from a mean become less obvious. The following table gives the sizes of the different groups 26 days after the beginning of the experiment:

		set I	set II
May 23.	Original size, average . . .	1.4 cm	2.05 cm
June 18.	Average size		
	control unfed	2.2 -	3.0 -
	liver	3.6 -	4.1 -
	muscle	3.5 -	3.9 -
	thymus	3.6 -	4.1 -
	adrenal	2.8 -	3.9 -
	hypophysis	2.8 -	— -
	testis	2.5 -	3.5 -
	ovary	2.1 -	3.3 -
June 14.	thyroid	1.1 -	1.8 -

Testis and ovary could not be fed regularly on account of the material lacking, so that these animals are not much ahead of the unfed control.

The thyroid fed animals had died on June 16 as fully developed frogs, though dwarfs in size; therefore their measurement on June 14 is given, on which day the fore legs in set I were also noticeable.

From the notes the following data may be given[1]):

May 23. Beginning of the experiment.

May 30 (up to this day food had been given 4 times). All thyroid II show hind legs.

June 4. 3 thymus II show hind legs.

June 5. The differences in size between the individual groups become marked, thyroid I and II are smaller and thymus I and II bigger than the other animals. The differentiation (limbs, form of the body etc.) is most evident in thyroid.

June 6. More thymus II and some of the other groups grow hind legs.

June 7. The differences in size and differentiation have become more striking. Thymus II are the biggest animals, they have, however, retained the typical form of tadpoles. Thyroid II begin to grow fore legs and to reduce their tails, their bodies are markedly frog-like.

June 8. All thyroid I grow hind legs. Thyroid II have become typical, but very small frogs and begin to jump. Some adrenal I

[1]) For the sake of simplicity, the organ given is used throughout the paper as an index of the respective group. I and II mean: size I and II. For instance, liver I means: tadpoles, size I, fed on liver, etc. Thymusthyroid means: tadpoles, fed first on thymus, later on thyroid.

and II show a somewhat lighter color than the rest of the tadpoles.

June 14. Thyroid II begin to die off. Thyroid I grow fore legs and start to absorb their tails.

June 16. Thyroid I die off. 2 thymus II grow fore legs — i. e. 9 days later than thyroid II. The lighter coloring of the adrenal I and II becomes more evident.

June 18. Thymus I and II are the biggest tadpoles of each set. The difference in length between thymus I and II and liver I and II is not marked, yet the thymus tadpoles are broader, have stronger legs etc.

June 20. The liver show a greenish coloring. Some thymus I and liver I grow hind legs, i. e. 12 days later than thyroid I.

June 23. Thymus II seem to show a retarded differentiation being behind liver II, muscle II and adrenal II.

June 25. This difference becomes more evident. All adrenal tadpoles show a color markedly lighter than that of the rest of the groups.

June 27. Thymus I are much bigger, yet far less differentiated than liver I and muscle I.

June 29. For several days no more of the thymus II have grown fore legs, while more and more of the liver II, muscle II, adrenal II and even some of the poorly fed testicle II and ovary II have done so.

July 2. Thymus I and II are very big and their color is very dark. No more thymus II develop fore legs, while of liver II, muscle II and adrenal II there is only one in each group without fore legs. Even of the poorly fed testis II and ovary II there are only 3, respectively 5 without them. Adrenal I grow hind legs, muscle I fore legs.

July 3. All liver II, muscle II and adrenal II tadpoles have grown fore legs.

July 8. The adrenal II frogs which on July 3 had been taken out of the water and placed on sand are now just as dark as the frogs of the other groups. There is only 1 of liver I and 3 of muscle I without fore legs, while there are still 9 of thymus II and 10 of thymus I without them, though the latter tadpoles are much bigger than the former.

July 9. Hypophysis I and testis I grow hind legs, adrenal I are the largest, almost as big as thymus I.

July 11. Adrenal I grow fore legs.

July 13. There are only 2 of muscle I without fore legs, while there are still 7 of thymus I, and 5 of thymus II lacking fore legs.

July 17. Some hypophysis I and testis I grow fore legs, the last muscle I grows fore legs, while there are still 3 of thymus I and 3 of thymus II without them. Hypophysis I tadpoles gradually become rather transparent, especially their heads. They show on the right side of their body a greenish swelling beneath the skin. The green color is also seen through on the right side of their bodies, yet there is no swelling there. The tails of some are twisted in a peculiar manner.

July 21. Hypophysis I begin to die one after the other without completing their metamorphosis. There are still 3 thymus I and 3 thymus II without fore legs, their bodies assume a very irregular shape and become very broad and bloated.

July 26. The last adrenal I grows fore legs.

Aug. 3. 1 more thymus I grows fore legs, there remain 2 without fore legs.

Aug. 4. 1 more thymus I grows fore legs, there remains 1 without them, 2 thymus II die without fore legs. One only survives.

Aug. 5. The last thymus I and thymus II die without fore legs.

Aug. 17. Some of the unfed control grow hind legs.

It is evident from the data just given that the thyroid and thymus tadpoles (Fig. 1 *a*—*b*, 2 *a*—*b*) reacted most peculiarly to their specific foods, while the liver, muscle and adrenal animals showed a more indifferent behavior. However, the very light coloring of the adrenal was striking as compared with the very dark color of the thymus and the dark greenish one of the liver tadpoles. It is highly probable that the light color of the adrenal is not the result of the feeding with adrenal, but was merely a contraction of the pigment cells due to the contents of the chromaffine cells going into solution (adrenalin reaction). The gradually developing transparency of the hypophysis fed tadpoles must also be mentioned as well as the fact that most of them died without completing their metamorphosis. The nature of the green swelling in their abdomen can only be determined by microscopic examination.

The results of the testis and ovary feeding are inconclusive since regular feeding was impossible on account of the difficulties met with in providing the food. The behavior of the other groups,

however, is characteristic, and there is also a definite control given, as the experiments were conducted on two different sets with corresponding results.

The quickest results were seen in the thyroid groups. While an increase in body size was lacking, the differentiation of the body was extremely rapid, both hind and fore legs appeared earlier than in any other group and the animals metamorphosed long before those fed on other substances. It was peculiar that every change in the body form set in almost simultaneously in all the animals of one set, so that the corresponding stages of development were reached within 24 hours or less; for instance, all had their hind or fore legs come out on the same day etc. In no other group could such a uniform development be observed. The only explanation of this can be the increased velocity of the differentiation processes. In the other groups only a few animals at first began to grow hind or fore legs, and often many weeks elapsed before all the others had reached the same stage in development. This is the natural course of events, since *a priori* not all tadpoles possessed the same vitality. The thyroid food, however, enacted such a strong accelerating influence on the body differentiation that the differences in time which existed in the development of the individual tadpoles were so reduced, that they hardly remained noticeable. The greatest difference in time, between the slowest and the most rapid differentiation of thyroid tadpoles of one set was less than one day.

The difference in time between the thyroid groups and those fed on other substances was as might be expected greater, the longer the treatment lasted. For instance, while only 5 days (May 30—June 4) lie between the appearance of the hind legs in thyroid II and thymus II, this interval in set I — I is the younger set, therefore was fed longer — is 12 days (June 8—June 20).

The precocious body differentiation of the thyroid fed tadpoles did not allow the animals to continue their growth, the result of the metamorphosis were therefore extremely small (pigmy-) frogs (Fig. 1*a*, 2*a*).

The feeding with thymus showed an influence on the development of the tadpoles, exactly the opposite of that caused by the thyroid diet. Its consequence was a prolonged increase in size beyond the normal, the metamorphosis, however, was much retarded or not completed at all as the animals died before that time. With this retarded development the individual differences, of course, were much

emphasized. Not all individuals of one set grew their hind or fore limbs on the same day, as in the thyroid groups, but there were intervals of days and weeks between the corresponding stages in different individuals. Those tadpoles that possessed the least amount of vital energy had to be fed longest. They were, therefore, longest under the influence of the retarding food.

The later an organ develops in normal ontogeny, for instance fore legs later than hind ones, the more its appearance was postponed by the thymus and accelerated by the thyroid diet. The hind legs of thyroid II and thymus II appeared at an interval of 5 days (May 30—June 4), for the fore legs the interval was 9 days (June 7—June 16). This can also be expressed in the opposite way: the younger a tadpole is at the beginning of the feeding, the greater is the retarding influence on development by the thymus treatment and the accelerating influence by the thyroid treatment. For instance, in the appearance of the hind limbs in thyroid II and thymus II (older set) the difference in time is only 5 days (May 30—June 4), while in thyroid I and thymus I (younger set) 12 days (June 8—June 20). In this comparison those thymus fed tadpoles with quickest differentiation, about 10%, were chosen. If all the thymus tadpoles were considered, the average difference in time would be much greater; for thymus I and II, although fed regularly and abundantly, needed about two months before all had completed their development, while all the other groups had metamorphosed long before.

Thus the influence of the thymus food was such that in the beginning it caused a rapid increase in body size, going beyond the normal, while later on it postponed the metamorphosis extremely. The color of the animals became very dark during the experiment. Those tadpoles most backward in development showed a clumsy bloated shape.

Experiment II.

A group of *Rana temporaria* tadpoles, originally selected for ovary feeding, had been fed only twice, May 23 and May 25, with that substance, after this time, up to the start of experiment II, July 6, through 43 days, they had starved. The short feeding of ovary was of so little influence, that these animals differed in no respect from the unfed control tadpoles (Fig. 3). From July 6 on a part of these tadpoles were fed on thyroid, another part on thymus. The differences in the results were most evident (Fig. 6 *a*, *b*) and corresponded to those of experiment I.

The diary reads as follows:

July 6. Start of the experiment. Average size 2.75 cm.

July 9. (after 3 days only) thyroid grow hind legs.

July 11. A difference in the sizes of thyroid and thymus is noticeable. 2 thymus grow hind legs.

July 12. Size of thyroid 2.6 cm, of thymus 3.2 cm. Thyroid assume frog-shape.

July 13. Thyroid grow fore legs and swim on their back.

July 14. Thyroid have completed their metamorphosis and begin to die.

July 17. Thymus are very big, entire length 3.7 cm (body 1.3 cm, tail 2.4 cm) and are very dark colored.

July 18. 2 thymus are still without hind legs. From to-day on these two will be fed on thyroid, so that the experiment now runs thus:

Thymus.	Thymusthyroid.
July 21.	After 3 days only! Appearance of hind legs.
July 22.	Body assumes frog-shape. The dark (thymus) color has disappeared.
July 23.	Fore legs! The animals swim on their back.
	Length of body 0.8 cm 0.9 cm - - tail 1,3 - 1.3 - entire length 2.1 cm 2.2 cm
July 24. Not until to-day 2 of this group grow fore legs, although on July 17 they were so much further along than those in the right column.	
Aug. 5. The last one completes its metamorphosis, 22 days later than thyroid, and 13 days later than thymusthyroid.	

Thus experiment II ends with the same results as experiment I. The feeding on thyroid causes an extremely rapid differentiation of the body with a complete suppression of growth (compare Fig. 3 and 6 *a*), the feeding on thymus furthers the growth (Fig. 6 *b*), but retards the differentiation. This is most strikingly seen in the sub-

experiment described in the right column. Those tadpoles which were backward most on July 17 metamorphose after having been fed on thyroid for only 5 days, sooner than those farthest along in development, which remained on thymus.

In this experiment the influence of the thyroid food made itself manifest after only 3 days. The reason for this might be that the animals had starved through 43 days and had thus become older without being able to develop. They were, one might say, in a condition of latent overripeness and the first application of food rapidly caused a further development.

Experiment III.

Taken alone experiment III would not allow of any conclusions, since it was done with only a few animals. Yet the results attained are absolutely the same as those of experiments I and II, and therefore furnish a confirmation of the latter.

Some control animals of experiment I which had been starving since the first feeding, May 23, through 51 days, were fed on thyroid from July 13, others on liver.

The experiment ran as follows:

Thyroid.	Liver.
July 13. Average size 2.4—2.6 cm	
July 18. Hind legs appear, tail gets shorter, body assumes frog-shape	
length of body 0.7 cm	0.9 cm
- - tail 0.8 -	1.9 -
entire length 1.5 cm	2.8 cm
July 19. They swim on their back.	
July 20. Fore legs just noticeable. They die off.	
July 25.	hind legs appear (7 days later than in thyroid!).
Aug. 16.	fore legs appear (17 days later than in thyroid!).

Experiment III again shows the extremely strong influence of the thyroid food in accelerating the development as compared with an indifferent food, as liver can be regarded. At the same time it confirms the above statement, that the differences in time between

corresponding stages of development become greater the later an organ appears in normal ontogeny. The time between the appearance of the hind legs in thyroid and liver was an interval of 7 days, while the interval between the appearance of the fore legs in the two sets was 17 days.

To gain a further control of the results of experiments I—III on *Rana temporaria*, a similar set of experiments was repeated on *Rana esculenta*.

Experiment IV.

Tadpoles of *Rana esculenta* were fed in groups of 20 on thyroid, thymus and liver and one group was left unfed. For the feeding on thyroid 3 groups of different sizes were used, the smallest ones in group I, the largest ones in III. Group II as well as the liver, thymus and control groups consisted of tadpoles of the intermediate size.

The differences in size at various times of the experiment are given in the following table:

		Control	Liver	Thymus	Thyroid I	Thyroid II	Thyroid III
July 6.	Size at the start of the experiment	cm 2.5—3.0			2.1	cm 2.7	3.3
July 17.	Length of body [1]	1.0	1.2	1.4	0.7	1.0	1.2
	- - tail	1.5	1.7	1.7	1.1	1.4	1.8
	entire length	2.5	2.9	3.1	1.8	2.4	3.0
July 21.	Length of body	1.0	1.2	1.4	0.7	1.0	1.1
	- - tail	1.5	1.7	1.8	1.0	1.1	1.6
	entire length	2.5	2.9	3.2	1.7	2.1	2.7
July 31.	Length of body	1.0	1.2	1.4			
	- - tail	1.5	1.8	2.1			
	entire length	2.5	3.0	3.5			
	breadth of body	—	0.6	0.7			
Aug. 10.	Length of body		1.2	1.5			
	- - tail		1.9	2.3			
	entire length		3.1	3.8			
	breadth of body		0.7	0.8			
	- - tail		0.4	0.7			

[1] At the beginning of the experiments only the entire length of the animals was measured. Later it was found better to determine the lengths of the body and tail separately.

	Liver	Thymus		
Aug. 16. Length of body	1.2	1.5		
- - tail	2.0	2.5		
entire length	3.2	4.0		
breadth of body	0.7	0.9		
		a	b	
Aug. 29. Length of body	1.3	1.6	1.9	a = smallest
- - tail	2.1	2.6	3.2	b = biggest
entire length	3.3	4.2	5.1	
breadth of body	0.7	0.9	1.0	

From the record of the experiment may be mentioned:

July 16. Liver are big and show a greenish color. Thymus are very big and dark. All thyroid II and III grow hind legs.

July 17. Thyroid I grow hind legs. Some thyroid II and III show buds of fore legs, thyroid II breathe very rapidly and swim on their backs. The bodies of all thyroid I—III assume frog-shape.

July 20. Thyroid II begin to die, they have typical frog-shape. Thyroid III swim on their backs.

July 21. All thyroid II are dead.

July 23. Thyroid I and III begin to die.

July 24. All are dead. Their bodies are typically frog-like.

July 31. The unfed control begin to die. The thymus lose their dark color and become lighter than liver.

Aug. 13. Liver begin to grow hind legs, 28 days later than thyroid.

Aug 15. Thymus begin to grow hind legs, 30 days later than thyroid.

Aug. 13—Sept. 15. Liver die one after the other without completing their metamorphosis.

Sept. 5—Sept. 7. Thymus die one after the other without completing their metamorphosis.

This experiment has therefore given the same results as those attained on *Rana temp.* The effect of the thyroid diet is again striking.

At present it is not clear, why the liver and thymus tadpoles in spite of the continual feeding (July 6—Sept. 7, Sept. 15) did not complete their metamorphosis, but died before. The only respect in which the *Rana esculenta* experiments differed from those on *temporaria* was the higher temperature of the water and air. The former were undertaken during the hottest period of the summer of 1911, while the latter had been completed before that time. However, it is unlikely that the rise in the temperature itself should have enacted such a retarding influence on the development of the tadpoles. One

should rather expect the contrary. Although the high temperature may not be directly injurious, it may indirectly create unfavorable conditions for artificially rearing the animals. The water in which the tadpoles were kept contained a large amount of organic substances constantly undergoing decomposition much more rapidly than on cooler days. Therefore the accelerating influence of the higher temperature may well have been counteracted by this process.

Still another reason may account for the delay in development. *Rana esculenta* is less fit than *temporaria* to be reared under artificial surroundings, therefore in general less resistent to aquarium conditions. Furthermore, it sometimes happens that under apparently favorable conditions *esculenta* tadpoles do not complete their metamorphosis before the following spring. BARFURTH and TORNIER state that overfeeding may postpone the metamorphosis.

The thyroid tadpoles did not succumb to any of the above mentioned influences. This can easily be explained by the fact that the thyroid treatment did not have to last very long on account of the immensely accelerating influence of that food.

During the first half of the experiment the thymus fed animals showed the same dark pigmentation as the *temporaria* did, later on this dark color disappeared and they became even lighter than the liver fed tadpoles.

Experiment V.

The aim of this experiment was to study the influence that a sudden change in the food given would have on the development of *Rana esculenta* tadpoles. For this purpose a set of animals which had been fed on liver since July 6 was on July 21 put on thymus-, another set on thyroid diet. The same was done with thymus fed animals which were put on liver and thymus respectively. Thyroid fed tadpoles for feeding on liver and thymus unfortunately could not be used. With other animals it was tried, however, to stop the rapid progress in differentiation after thyroid diet by giving liver or thymus, but without results.

a. Liver fed tadpoles, put on thymus or thyroid diet on July 21.

	Liverthymus.		Liverthyroid.
	Average size:		
July 21.	Length of body	1.2 cm	1.2 cm
	- - tail	1.7 -	1.7 -
	entire length	2.9 cm	2.9 cm

Liverthymus.	Liverthyroid.
July 24.	After 3 days feeding! hind legs appear.
July 27.	Frog-shape is noticeable.
Length of body 1.2 cm	1.1 cm
- - tail 2.1 -	1.6 -
entire length 3.3 cm	2.7 cm
July 31. Length of body 1.3 cm	1.0 cm
- - tail 2.3 -	1.4 -
entire length 3.6 cm	2.4 cm
breadth of body 0.75 -	0.6 -, length of legs 0.3 cm.
	Swim on the back, air vesicles in the gill region, begin to die off.
(continued unter c.)	

b. Thymus fed tadpoles, put on liver or thyroid diet on July 21.

Thymusliver.	Thymusthyroid.
July 21. Length of body 1.4 cm	1.4 cm
- - tail 1.8 -	1.8 -
entire length 3.2 cm	3.2 cm
July 24.	After 3 days feeding! hind legs appear.
July 27.	Frog-shape is noticeable.
Length of body 1.4 cm	1.3 cm
- - tail 2.1 -	1.8 -
entire length 3.5 cm	3.1 cm
July 30. Length of body 1.4 cm	1.1 cm
- - tail 2.1 -	1.5 -
entire length 3.5 cm	2.6 cm
breadth of body 0.7 -	0,6 -, length of legs 0.6 cm.
	Swim on the back, air vesicles in the gill region.
Aug. 2.	Begin to die. A few have the buds of the fore legs out.
Aug. 3.	The last ones die.
	Length of body 1.0 cm
	- - tail 1.5 -
	entire length 2.5 cm
	breadth 0.6 -
(continued under c.)	length of legs 0.6 -

c. Continuation of the left columns of the above tables. A comparison of liverthymus and thymusliver.

	Liverthymus.	Thymusliver.
July 21.	Length of body 1.2 cm	1.4 cm
	- - tail 1.7 -	1.8 -
	entire length 2.9 cm	3.2 cm
July 27.	Length of body 1.2 cm	1.4 cm
	- - tail 2.1 -	2.1 -
	entire length 3.3 cm	3.5 cm
July 31.	Length of body 1.3 cm	1.4 cm
	- - tail 2.3 -	2.2 -
	entire length 3.6 cm	3.6 cm
	breadth of body 0.75 -	0.7 -
Aug. 5.	Length of body 1.45 cm	1.4 cm
	- - tail 2.5 -	2.3 -
	entire length 3.95 cm	3.7 cm
	breadth of body 0.8 -	0.7 -
Aug. 10.	Length of body 1.5 cm	1.4 cm
	- - tail 2.6 -	2.3 -
	entire length 4.1 cm	3.7 cm
	breadth of body 0.8 -	0.7 -
Aug. 13.	Hind legs appear, 20 days later than in thyroid (compare the right columns of a and b).	
Aug. 16.	Length of body 1.6 cm	1.4 cm
	- - tail 2.8 -	2.3 -
	entire length 4.4 cm	3.7 cm
	breadth of body 0.8 -	0.7 -
Sept. 2.		Begin to die without developing fore legs.
Sept. 20.	Begin to die without developing fore legs.	

Experiment V shows that a thyroid diet, started even at an advanced stage of differentiation and after other substances have been fed, is able to influence the further development intensely. It seems of little importance, which substances were fed before the thyroid, except that the relative sizes are different. The liverthyroid went almost parallel with the thymusthyroid (Fig. 8*a*, *b*). Some minute differences, however, were noticeable, yet further experiments with a combined

diet will have to determine their importance. Some of the thymus-thyroid, for instance, showed buds of the fore legs, while the liver-thyroid died before that stage showing characteristic responses to thyroid. These features as swimming on the back, formation of air bubbles in the gill region and others will be discussed later.

The liverthyroid and thymusthyroid were far ahead of the liver-thymus and thymusliver and also of the liver and thymus of experiment IV.

The liverthymus and the thymusliver ran almost parallel with the exception that the liverthymus grew bigger than the thymus-liver. The thymus diet, therefore, furthers growth even, when it is given at an advanced stage of differentiation, but apparently less than when given to younger animals.

The following comparison of tables IV and V is interesting: liverthymus Vc become gradually larger than liver IV, thymusliver Vc smaller than thymus IV. The thymus food thus seems to act differently at different ages, and it may be possible to find a time or stage for its optimum influence such as is also surmised for the thyroid diet.

In liver IV the hind legs appeared on August 13, in thymus IV on August 15. This difference in time is rather small and further experiments must show, whether or not it is significant. At any rate, this observation agrees with those made on *Rana temporaria*, which showed that the thymus food retarded the development. Liverthymus V and thymusliver V grew their hind legs on August 13, i. e. on the same day as liver IV. Thus the partial feeding on thymus seems not to have caused the same delay in development as the exclusive thymus diet. However, a difference of only 2 days, observed on one set of animals does not allow of conclusions.

Experiment VI.

This experiment can be regarded as a supplement to experiment V, at the same time it furnishes a further confirmation of the results of former thyroid feedings. Tadpoles that had been fed on liver and thymus 15 days longer than the corresponding groups of experiment V, thus were 15 days older, were put on the thyroid diet on August 5.

This last experiment shows that the thyroid when food given even at a very advanced stage of differentiation can cause an accelerated development. 5 days after the beginning of the experiment hind legs appear, this is still 3 and 5 days sooner than in the control animals liver IV and thymus IV. The effect of the previous feeding on different

	Liverthyroid.	Thymusthyroid.
Aug. 5.	Length of body 1.2 cm	1.4 cm
	- - tail 1.9 -	2.2 -
	entire length 3.1 cm	3.6 cm
	breadth of body 0.6 -	0.8 -
Aug. 10.	Length of body 1.2 cm	1.3 cm
	- - tail 1.8 -	2.1 -
	breadth of body 0.6 cm	0.7 cm
	Hind legs appear after 5 days feeding. (The liver IV and thymus IV do not grow them until August 13 and August 15.)	
Aug. 12.	Frog-shape is noticeable.	
Aug. 14.	Swim on the back.	
Aug. 15.	2 grow fore legs.	
Aug. 16.	These two (a, b) die, the rest grow fore legs	
	a b rest	
	length of body 0.9 1.05 0.9 cm	1.15 cm
	- - tail 1.2 1.0 1.2 -	1.7 -
	entire length 2.1 2.05 2.1 cm	2.85 cm
	breadth of body 0.5 cm	0.7 -
Aug. 17.	Begin to die off.	Swim on the back.
Aug. 18.		1 grows fore legs.
Aug. 19.	Last ones die	Last ones die.
	smallest largest	smallest largest
	length of body 0.8 cm 0,9 cm	1.0 cm 1.1 cm
	- - tail 0.6 - 0.9 -	1.2 - 1.4 -
	entire length 1.4 cm 1.8 cm	2.2 cm 2.5 cm
	breadth of body 0.5 - 0.5 -	0.65 - 0.7 -

substances before the thyroid diet here also manifests itself in the different sizes of the animals. During the entire experiment thymusthyroid remain bigger than liverthyroid; on the other hand liverthyroid develop quicker than thymusthyroid, which is suggested also by experiment V. If further experiments of this kind give similar results, we shall have additional evidence, that thymus food postpones the metamorphosis. In fact, at the beginning of experiment VI the liver IV must have been ahead of thymus IV, although macroscopically the difference was not evident; for liver IV grew their hind limbs on August 13 and thymus IV on August 15.

General discussion.

The most striking and at the same time unquestionable results were attained by thyroid feeding. They were the same in all experiments. The influence of the thyroid food was such that it stopped any further growth but on the contrary led to an abnormal diminution of the size in the animals treated, while simultaneously it accelerated the differentiation of the body immensely and brought it to a premature end. It was of little importance, at which stage of differentiation the thyroid diet began or which kind of food had been given before. Under all circumstances the influence of the thyroid food became noticeable in a very short time.

This influence must have been very strong, as can be concluded from two kinds of observations. First, within a very short time, 3—5 days, after the beginning of the experiments changes in the outer features of the animals were noticeable; second, the influence on all tadpoles of one group was uniform and rather parallel. While, for instance, in other groups not fed on thyroid the influence of the food became evident gradually, without abolishing the individual differences, so that the individuals of one group grew their hind legs, fore legs etc. one after the other, often at intervals of many days, the thyroid diet, on the other hand, brought all the animals of one group within a few hours, not more than 24, to the same stage of development. However, it cannot be said that the individual differences were entirely abolished. The measurable signs of these differences, the intervals between the corresponding phases of development, were greatly reduced since the entire period of development was much shortened.

One of the most peculiar features is that the time at which the feeding begins is of no importance as regards its results. The stages of development of the animals to be treated may be chosen, but always the same results will be obtained. Animals in different stages of development, others that had starved for many weeks, and still others that had before been fed on other substances were placed on thyroid diet with exactly the same results: within a few days the rapid differentiation of the body began. Thus extremely young or very old tadpoles could be forced to undergo their methamorphosis quickly. The lower and upper limit of age for the start of a successful thyroid diet will be determined later. The upper limit is probably the time shortly before completing their metamorphosis, when the tadpoles stop feeding in general. How near to the time of hatching

the lower limit can be brought further experiments will show[1]). The tadpoles that were available for the experiments here recorded had been hatched for some weeks.

The second influence of the thyroid diet, the suppression of growth, is merely the consequence of the precocious development, and this in turn seems to be caused by the well known activity of the thyroid agens to stimulate metabolism. The thyroid agens accelerates the metabolism which leads to a rapid reduction of the larval organs and thus to a premature metamorphosis. As soon as thyroid food is given the differentiation of the body begins. Hand in hand with the progressing metamorphosis goes, more than in the case in normal development, a reduction of the body mass (resorption of the tail, loss of water, therefore an increasing compactness of the body etc.) The outcome of such precocious metamorphosises are then very small (pigmy) frogs. This mass reduction was especially striking in the experiments on *Rana esculenta*.

The thyroid showed still other peculiar influences on the behavior of the tadpoles. Towards the end of the metamorphosis the animals hardly moved about in the water. They were always lying quietly, generally on their backs. When disturbed they would move for a few seconds in a somewhat convulsive manner and then drop again to the bottom of the dish, while tadpoles fed on other material would swim about for a long time. The reason for this may be that the thyroid fed tadpoles always began to reduce their tail before the extremities were at all or sufficiently strongly developed. The extremities, even if fully developed, were always extremely thin, merely thread-like (Fig. 6 *a*), and could hardly be used for swimming a long time.

At one time *Rana esculenta* tadpoles of the different groups were placed in small dishes with equal quantities of water, to which equal amounts (about 5 drops) of chloroform had been added. This was done so as to be able to photograph the animals. All tadpoles remained the same length of time in the mixture. All animals survived the narcosis very well except the thyroid fed ones which died in it.

At another time *Rana temporaria* tadpoles were taken out of the water and placed on wet filter paper to photograph them. During this procedure, which of course was somewhat rough, the thyroid

[1]) In recent experiments (1912) which will be discussed in a later paper I succeeded in forcing *Rana temporaria* tadpoles to grow fore legs as early as 15 (!) days after leaving the egg.

died, while the others stood it. Thus in different ways it was seen that the thyroid fed tadpoles possessed far less resistance against noxious influences than the others, as if the thyroid food had weakened their systems enormously. One cannot, however, speak of a poisoning of their body in the true sense, since that would not have allowed the rapid progress in development.

In the tables given above several dates are mentioned at which the animals began to swim on their backs. This, too, is one of the features observed only in thyroid tadpoles. Before the animals completed their metamorphosis, about 3—4 days previous, they began lying on their backs and floated passively on the surface of the water. They breathed very heavily and rapidly. Even when disturbed and swimming actively they did not usually turn over. It seemed as if the animals were passively forced to take this peculiar position; as under the skin in the gill region there were always one or two air bubbles visible, as if during the closure of the gill opening air had been enclosed. If the animals did not die these air bubbles were usually absorbed after which the animals assumed a normal position. It was seen that the swimming on the back always began shortly before the completion of the metamorphosis and its early appearance was watched.

The influence of the thymus diet on the development of the tadpoles was as evident as that of the thyroid, but less striking. The thymus food caused an accelerated growth beyond the normal (giant tadpoles) and at the same time it retarded or completely suppressed the differentiation of the body. In doing so individual differences were very much emphasized, so that an interval of several weeks elapsed between the metamorphosis of the first and the last tadpole, while in normal development the difference amounted to days only. The strongest tadpoles or better those which at the start of the feeding had progressed most in their development were best able to keep pace with the control. Those, however, which were backward in their development at the time the thymus diet began stayed much behind the control, since they were attacked by the thymus at a less advanced stage of differentiation, and further because they remained longest on thymus diet.

The thyroid and thymus diets were thus diametrically opposite in their influences. Their relative action, however, corresponds with the views held regarding the physiological properties of these organs.

Experiments of the kind discussed in this paper may perhaps give a direction for further studies towards a rational application of thymus and thyroid preparations.

It is not the purpose of this experimental paper to discuss the extensive literature on the functional and therapeutic importance of the organs with an internal secretion. Reference is simply made to the numerous papers in which the therapeutic value of thyroid preparations for the stimulation of metabolism and ossification, and the influence of the thymus on growth in the early periods of individual life are being discussed. A list of them will be found at the end of this paper.

Liver and muscle were about equal in their action on development and did not seem to influence especially the normal progress of differentiation. Since so far they appeared to be indifferent food stuffs the tadpoles fed on liver or muscle were regarded as a control to the other feedings. However, under natural conditions the animals have a food supply quite different from a constant meat diet, yet for various reasons it was impossible to study the development of control animals on a more vegetal or mixed diet[1]).

The tadpoles fed on adrenal[2]) developed somewhat slower than those fed on liver or muscle, otherwise quite normally. The outcome of the metamorphosises were especially large and strongly developed frogs (Fig. 5*b*).

A prolonged diet of hypophysis did not force the animals to complete their methamorphosis. They all died before that stage. No conclusions can be drawn from this fact, since these tadpoles were not fed as regularly as the others on account of great difficulties encountered in providing the food[3]). The feeding on testis and ovary was also unsatisfactory for the same reason.

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[1]) Such experiments are now being done, spring 1912, and they will be discussed in a later paper. The difference in macroscopic development between a vegetal and liver or muscle diet is slight.

[2]) Experiments on feeding adrenal cortex and medulla separately will be discussed in a later paper.

[3]) More extensive experiments on feeding the two lobes of the hypophysis will be discussed in a later paper. So far they do not confirm the above statement.

Preliminary experiments were also undertaken on *Rana esculenta* to study the influence of different diets on regenerating animals. So much can be said that of tadpoles which hat a piece of their tail amputated the thymus fed ones regenerated quickest, while the thyroid fed ones, although they did regenerate a part, showed the typical precocious metamorphosis. In one experiment the average length of the regenerated part of the tail was: in thymus 3.5 mm, in thyroid 3.2 mm, in liver 2.9 mm; in another experiment: liver 3.1 mm, thymus 4.6 mm; later liver 6 mm, thymus 9 mm. Regeneration of the tail begins even when the animals are near the point where they resorb their larval organs, otherwise the thyroid fed ones would not have regenerated. BARFURTH showed that *Rana fusca* tadpoles which metamorphosed even 2 or 3 days after the operation tried to regenerate the amputated part of their tails.

The influence of the different food stuffs on the pigmentation has been mentioned before. The animals were kept under the same conditions of light and temperature and in the same kind of dishes. The position of the dishes was changed daily in a certain rotation so that the minute differences in light and temperature were abolished as much as possible.

The liver fed tadpoles were rather dark, gradually assuming a greenish tint. The thymus fed tadpoles of *Rana temporaria* grew extremely dark with the progress of the experiments until they became almost black; those of *R. esculenta* grew dark in the beginning, later, however, they became lighter. The adrenal fed tadpoles after 3—4 weeks became extremely light in color. Those fed on hypophysis lost their pigment more and more and became almost transparent, but this may have been the consequence of the irregular feeding.

TORNIER has studied the influence of varying quantities of food on the pigmentation of *Pelobates fuscus* tadpoles and found that a minimum food ratio gives albinotic, a maximum ratio highly melanotic larvae and frogs. So the melanism of the thymus fed tadpoles may have been partly caused by an overrich diet, yet they were much darker than those in the other groups, although all were fed sufficiently well. Why the *Rana esculenta* larvae which in the first weeks of the experiments were as melanotic as the *temporaria*, later lost their dark appearance, cannot be explained at present. Very minute differences in temperature, as KAMMERER points out, may easily cause a change in pigmentation.

The very dark and the very light adrenal (cortex and medulla) tadpoles seem, roughly estimated, to have possessed equal amounts of pigment. In the thymus fed animals the pigment cells were spread out very much in a star-like manner, in the adrenal fed ones they were completely contracted. Former experiments with adrenalin would warrant the suggestion that the extract from the chromaffine cells of the medulla which dissolved in the water caused the pigment cells to contract[1]).

The histogenetic processes must have been influenced very much by the different diets. The investigation of the thyroid and thymus fed material promises especially interesting results. The report on this topic will be given later.

More experiments, especially with mixed diets, are necessary to clear up all the questions concerned in this discussion. At any rate, these experiments may open a new and extensive field of work in experimental morphology, in which success is rather certain.

At present one fact alone deserves notice, that the food stuffs given fresh were able to pass the stomach without losing at least some of their specific properties. It still remains an open question, whether their action, after they have passed the intestinal canal, is entirely the same as that which they exert as functionating organs. Before this question is solved, no conclusions can be made on the rôle of these organs in the household of the body. However, so far it has been shown that a diet on thyroid substance or the application of thyroid tablets can to a certain degree substitute the normal function of the thyroid gland. — It must also be kept in mind that mammalian organs were fed to amphibians.

Summary.

A number of mammalian organs, especially those with an internal secretion, thyroid, thymus, adrenal, testis, ovary, hypophysis, liver, muscle etc. were given as food to tadpoles of *Rana temporaria* and *esculenta*. It was seen that each organ exerted a certain influence on growth and differentiation of the animals. Most striking was the

[1]) Compare: Lieben, S., list of literature. Recent (1912) experiments, however, so far indicate that the feeding on adrenal cortex causes a much lighter pigmentation than an adrenal medulla diet.

influence of the thyroid food. It caused a precocious differentiation of the body, but suppressed further growth. The tadpoles began to metamorphose a few days after the first application of the thyroid and weeks before the control animals did so. The influence of the thymus was quite the opposite, especially during the first days of its application it caused a rapid growth of the animals, but postponed the final metamorphosis or suppressed it completely. The action of the other organs must be studied further before definite statements can be made. The thymus diet gave very dark, melanotic tadpoles, the adrenal diet extremely light albinos, the liver diet dark ones with a greenish tint.

Zusammenfassung.

Verschiedene Säugetierorgane, namentlich solche mit innerer Secretion, Thyreoidea, Thymus, Nebenniere, Hoden, Eierstock, Hypophyse, Leber, Muskel usw. wurden an Kaulquappen von *Rana temporaria* und *esculenta* verfüttert. Jede Fütterung übte einen andern Einfluß auf das Wachstum und die Differenzierung der Tiere aus. Äußerst auffallend war die Wirkung der Schilddrüsennahrung. Sie verursachte eine rapide Körperdifferenzierung, die zu einer vorzeitigen Metamorphose führte, wobei aber jedes Weiterwachstum unterdrückt wurde. Die Kaulquappen begannen ihre Metamorphose wenige Tage nach der ersten Schilddrüsendosis und um Wochen früher als die Kontrolltiere. Der Einfluß der Thymusnahrung war gerade entgegengesetzt. Sie bewirkte namentlich in den ersten Tagen ein schnelles Wachstum der behandelten Tiere, schob aber die Metamorphose immer weiter hinaus oder unterdrückte sie gänzlich. Der Einfluß der übrigen Organe muß noch weiter studiert werden. Die Thymusverfütterung ergab tief dunkel, fast schwarz gefärbte Quappen, die Nebenniere ganz lichte, albinotische Tiere, die Leber dunkle, mit einem Stich ins Grünliche.

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Explanation of Figures.

Plate IX.

Fig. 1 *a*—*e*. *Rana temporaria* set I (smaller size), photographed June 11 1911. Natural size. *a* tadpoles fed on thyroid, already changing into frogs. Tail is shortening, fore legs appear. *b* tadpoles fed on thymus, *c* on liver, *d* on muscle, *e* on adrenal.

Fig. 2 *a*—*e*. *Rana temporaria* set II (larger size), photographed June 11 1911. Natural size. *a*—*e* as in Fig. 1. The thyroid fed tadpoles have all metamorphosed, the tail in some has almost disappeared, fore legs are well developed.

Fig. 3. *Rana temporaria* tadpoles that had been used as an unfed control in experiment I, thus starved till July 13. Photogr. July 13. Nat. size.

Fig. 4. The same tadpoles as in Fig. 3, photogr. 7 days later, July 20. *a* had been fed in the mean-time on thyroid, and are already metamorphosing into pigmy frogs. *b* had been fed on liver. These tadpoles do not metamorphose until 19 days later.

Fig. 5. *Rana temporaria* set I frogs. Photogr. July 25. Natural size. *a* fed from the beginning of experiment I on adrenal. *b* one of the tadpoles that originally had starved. Their size on July 6 was that of the tadpoles in Fig. 3. From July 6 to July 17 these tadpoles, 5 *b*, were fed on thymus, from July 18 on thyroid. On July 21 hind legs appeared, July 23 fore legs. Notice the small body and the much shortened tail of a frog metamorphosing under thyroid influence, while the adrenal frogs, 5 *a*, at the time of metamorphosis are large and still have their long tadpole tails.

Fig. 6. *Rana temporaria* set I, photogr. July 13 1911. Natural size. Animals that originally had starved. Their size on July 6 was that of the tadpoles in Fig. 3. From July 6 to July 13. *a* were fed on thyroid, *b* on thymus. *a* are changing into pigmy frogs, fore legs appear, tail shortens, *b* are still huge tadpoles. Compare also these thymus tadpoles with Fig. 4 *b* fed for the same time on liver.

The animals in Fig. 4—6 are all of the same age and the same original size, set I, but fed on different organs.

Fig. 7. *Rana esculenta*, photogr. August 9, nat. size. *a* fed on thymus since July 6, *b* fed on liver since July 6, *c* on thymus from July 6 to July 20, on liver since July 21, *d* fed on liver from July 6 to July 20, on thymus since July 21.

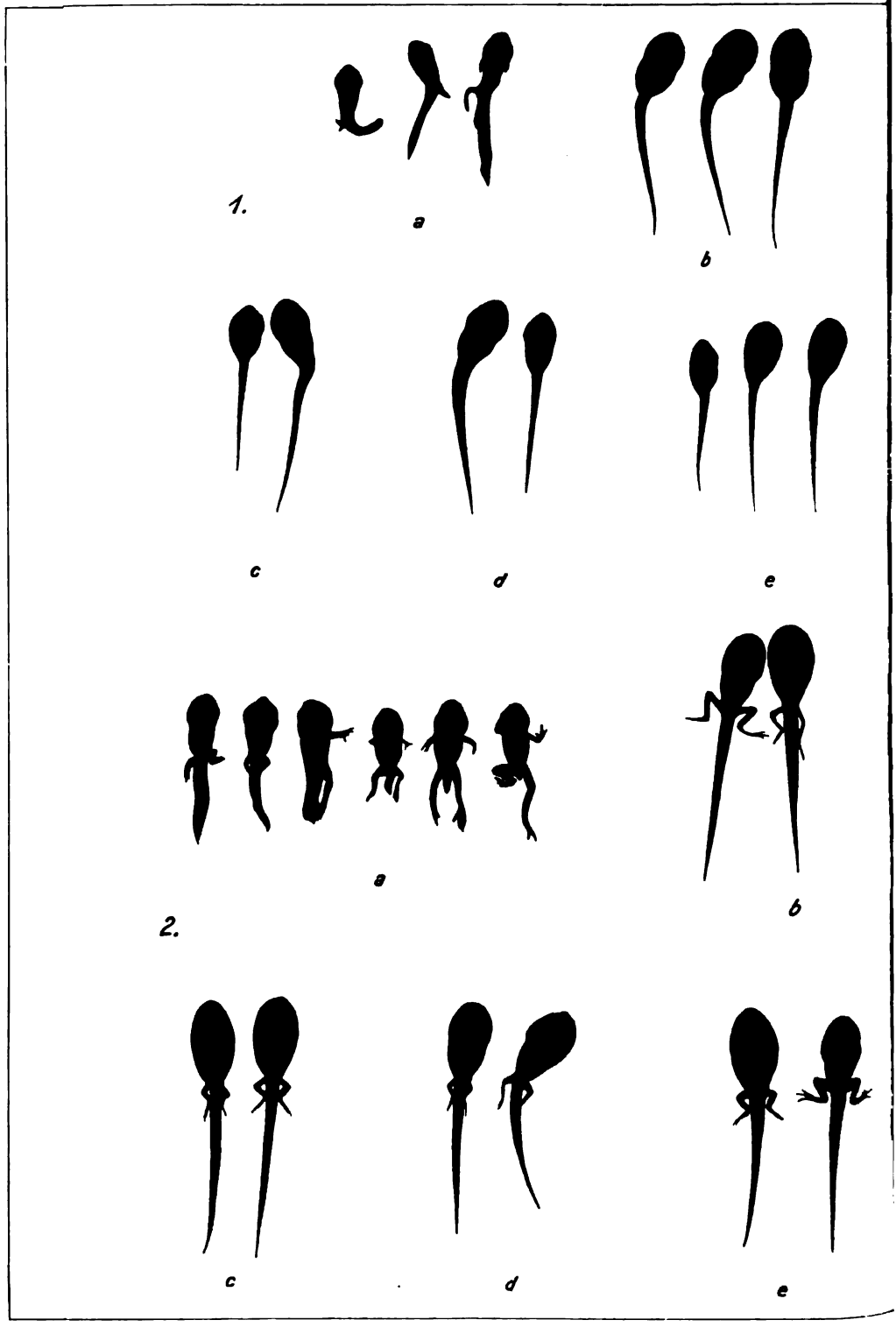
a and *c*, *d* which either entirely, *a*, or at a time of their development, *c*, *d*, were fed on thymus, are much bigger than those fed on liver only, *b*.

Fig. 8. *Rana esculenta*, photogr. August 19 1911, nat. size. The animals were dead, when being photographed, therefore the curved tails. *a* fed from July 6 to August 5 on thymus, from August 6 to August 18 on thyroid. *b* fed from July 6 to August 5 on liver, from August 6 to August 16 on thyroid.

The animals in Fig. 7 and 8 are all of the same age, but fed on different substances, and were photographed on the same day.

Anmerkung. Vorliegende Untersuchungen wurden im Sommer 1911 in meinem Institute durchgeführt. Das Manuskript war bereits im Dezember 1911 druckfertig. Eine schwere Krankheit, die mich lange zur Untätigkeit zwang, hat die Veröffentlichung verzögert.

Prag, Mai 1912. Prof. Alfred Kohn.



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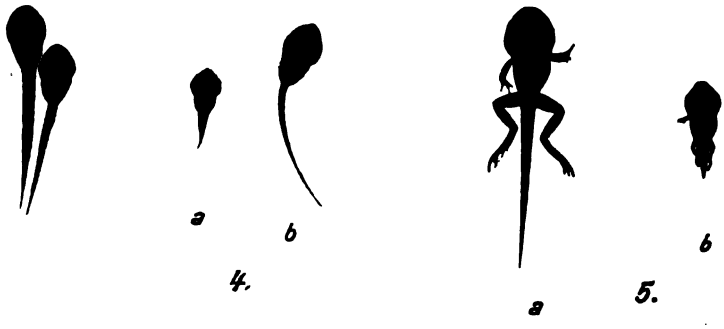

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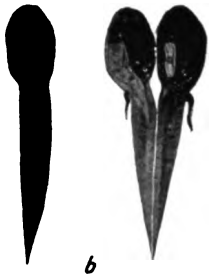

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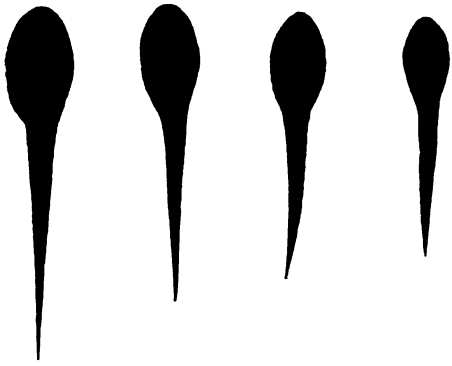

7.

a c d b

a

8.

b

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THE BEHAVIOR AND RELATIONS OF LIVING CONNECTIVE TISSUE CELLS IN THE FINS OF FISH EMBRYOS WITH SPECIAL REFERENCE TO THE HISTOGENESIS OF THE COLLAGINOUS OR WHITE FIBERS

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TEN FIGURES

In the process of connective tissue development the cells first arise, the fibers later appear. This sequence is established beyond controversy. The ontogenetic relation of cell and fiber is not, however, so thoroughly established. The theories advanced may be grouped under three heads: (1) Intra-cellular origin, the cells may transform into fibers (Schwann, Valentin, Boll, Flemming, Spuler, Livini). (2) Extra-cellular origin, the fibers arise in the intercellular substance by its fibrillation, or possibly as a secretion from the cells (Henle, Merkel, Virchow, Kölliker). (3) Epicellular origin, the fibers form in an ectoplasm at the surface of the cell (Schultze, Hansen, Golowinski, Mall).

These theories have all been primarily founded upon the results of examination of 'fixed' or 'killed' tissue, or upon the study of fresh teased tissue. Living connective tissue has been studied in the mesentery of the frog and other animals under conditions which are accompanied by marked inflammatory reaction and certain stages of the formation of exudates and of scar tissue have been thus investigated, and more recently movements of connective tissue cells have been observed in tissue cultures but so far as I know the theories of the histogenesis of connective tissue have not been examined with reference to the behavior of living cells under normal conditions.

Living connective tissue cells have been seen in tissue cultures by Harrison, Burrows, Carrel and Burrows, Margaret R. and W. H. Lewis and others, to exhibit a certain motility, and Harrison has recently emphasized the stereotropic tendency of connective tissue cells in cultures when in contact with foreign surfaces, glass, spider-web, etc. But so far as I know, the histogenesis of connective tissue fibers has not been so studied, and at best the culture method is open to some criticism on the ground that while the connective tissue cells are undoubtedly alive and active, yet they exist under very unusual, if not abnormal, conditions whose effects have not yet been subjected to complete analysis. Under these conditions the behavior of the connective tissue elements while probably similar, is not certainly in exact conformity with that of the tissue within the embryo.

In order that deductions based upon these several methods of examination be adequately controlled it appeared desirable that developing connective tissue be studied in the living animal under conditions which were in every respect normal, or which, at least, resulted in no inflammatory reaction. In mammals this endeavor is fraught with considerable difficulty owing to the size of the mammalian embryo and the depth beneath other tissues, often not transparent, at which the connective tissue lies.

During the past summer I had the opportunity, through the courtesy of the Marine Biological Laboratory at Woods Hole, of studying connective tissue in the fins of living fish embryos under conditions which were wholly normal and unaccompanied by any evidence of inflammatory reaction.

If a free swimming Fundulus embryo is placed on a hollow ground slide it will continue to swim, often actively, and its heart beat and circulation are maintained. It may be observed for some minutes and at the end of observation may be returned to the aquarium to continue an uneventful existence for hours or days thereafter. If a drop of chlorotone is added, or frequently without its addition, the fish will remain quiet for some minutes, thus permitting continued observation of connective tissue cells in his semitransparent fins. Certainly cells studied under these conditions are open to no criticism of abnormality.

The viability of the animals is unaltered for I have kept them for several days after such observation without any indication of decreased activity on their part. Even embryos which have been quieted by chlorotone, as well as those immersed for hours in a solution of Bismark brown in sea water, I have resuscitated and kept alive and in an apparently normal and usual condition for two or three days; they could easily have been kept longer had it seemed advisable.

The tissue selected for observation was in the fins of free swimming pelagic and Fundulus embryos. The embryos studied were chiefly of Fundulus and varied from 5 to 20 mm. in total length. The most favorable subjects were from the time of hatching, 5 to 6 mm., up to 12 mm. in length. The pectoral and caudal fins were usually selected as most available for observation. In such embryos the fin consists of a central frame work formed by the jointed rays, lepidotrichia, with their attached muscles, and a superficial integument of pavement epithelium with its subjacent basement membrane. The finer fin rays, actinotrichia, continue the jointed rays to the margin of the fin. The fin at this stage is very thin and the epidermis lies almost in contact with the fin rays. But between adjacent rays is an interval which lodges on either side the afferent and efferent blood vessels, bordered by chromatophores, and between them a loose mass of mesenchymal connective tissue in which the cells may be readily observed.

In embryos 5 to 6 mm. long the connective tissue in the pectoral fins consists chiefly of a mass of round cells confined to the proximal portion, and beyond this mass a distal fringe or 'skirmish line' of scattered stellate cells. In the unpaired fins, which are less advanced in their development only the scattered stellate cells are represented, the invasion of the round cell mass having not yet occurred. In later stages, as in the caudal fin of the same embryo, the zone of round cells has advanced distalward among the actinotrichia nearly to the fin margin, leaving behind between the lepidotrichia an area of more mature cells, stellate and spindle, and a few fine fibers well separated by broad spaces occupied by tissue fluids. The spindle cells and fibers preponderate in the

proximal, the round cells in the distal zone of the fin. Hence, one follows the sequence of development in passing from the distal toward the proximal portion of such a fin. Older embryos show the same zones of transition but in them the formation of fibers in the proximal region is more advanced.

In mammalian tissue one finds three stages in the histogenesis of connective tissue, a primitive cellular stage, a syncytial stage, and a fibrous stage. The first is characterized by the predominance of round cells, the second by stellate, the third by spindle and lamellar cells. The same succession of cell types is present in the fins of embryo fish and there is a corresponding succession of histogenic stages. Fibers do not appear prior to the appearance of cellular processes. Fine fibers appear coincidently with stellate cells, coarse fibers and fiber bundles develop later.

In the distal portion of the fin fine fibers first appear in the round cell area coincidently with the transition from round to early stellate forms. At exactly this period I have observed the first indication of motion, the throwing out of pseudopods by the round cells, in the connective tissue cells of the living embryo. Fig. 1 shows such changes in two cells on the border of the round cell area near the posterior end of the ventral fin. There is at this time relatively little locomotion, as is shown in the figure by referring the position of the cells *a* and *b* to the relatively fixed point, a prominence on the margin of an adjacent chromatophore (*ch*).

The first appearance of fibers in the distal portions of the fins has been very properly connected by Harrison, and by Goodrich with the origin of the dermal fin rays from the 'scleroblast' cells which closely resemble the connective tissue cells and like them are of mesodermal origin. In the region of the actinotrichia in the distal portion of the fin, it is difficult to distinguish between the early forms of these coarse fibers and the true connective tissue fibers, but the actinotrichia are confined to the region of the last one or two joints of the jointed fin rays, and there they project, as Goodrich has shown, from between the two opposed dermal plates which form the distal section of the jointed fin ray. If therefore one studies a region proximal to the last section and

selects the interval between the jointed rays the primitive actinotrichia are thereby excluded.

In such portions of the caudal fins of 6 mm. embryos, and in equivalent places in later stages, are typical connective tissue fibers mostly occurring as coarse longitudinal bundles with fine oblique anastomoses. Single fibers occur in the intervals of the coarser bundles. It is along these fibers and fiber bundles that the stellate and spindle cells are disposed. These cells are readily seen in the living fish, though the ease of observation is subject to much variation in different individuals and to a less extent in different portions of the same embryo.

My observations were made on living embryos immersed in sea water, some with, some without the addition of chlorotone. In some cases a few drops of a saturated solution of Bismark brown were added to the sea water in which the fish was kept, the effect of which after a time was to slightly increase the color contrast between the connective tissue cells and surrounding structures. The stain seemed almost inocuous, for fish could be kept in it for several days without apparent effect on their vitality. Many of the fish thus examined were later killed, and the fins stained and mounted in toto, or sectioned. The various cell types seen in life were readily recognizable in corresponding locations in the stained preparations.

It is in life difficult or impossible to distinguish between the spindle and lamellar types, though in 'fixed' tissue they may be morphologically distinct. In the living animal one can see a stellate or a spindle cell elongate, approach and flatten itself against a connective tissue fiber or fiber bundle, becoming sometimes so attenuated as to be scarcely distinguishable from the fiber against which it lies; it may at any time acquire increased thickness. Such a relation to a connective tissue fiber is shown by the cell *b* in fig. 2. The relationship is again exhibited by the two cells shown in fig. 3, one of which *a*, approached a small fiber bundle, became flattened against it, then rotated to the opposite side of the fiber at 9.30 A.M., and later freed itself from the contact. Its locomotion can be observed in relation to the chromatophore (*ch*) which served as a fairly fixed point. Similar

cells are frequently seen flattened against the surface of fiber bundles, blood-vessels, or fin-rays, and exhibiting a slow stereotropic locomotion. Many of these cells would seem to be identical with those which in stained preparations we are accustomed to call lamellar cells.

That connective tissue cells exhibit a certain amount of motion is no new observation. It has been well known since the inflammatory reaction to injury or infection was studied in the mesentery by Arnold and others. I have observed that the extent and rapidity of the motion varies with the cell type. The round cell, or primitive type, presents relatively little motion, it being limited, so far as I have observed, to the very slow projection and retraction of minute pseudopods. Even this evidence of activity seems rather to be limited to those later phases of the cellular stage which foreshadow the transformation of the round cells to the stellate type of the succeeding stage. This transformation is indicated by the fact that the motion is more noticeable near the border of the round cell area than in its interior, and also because at the extreme margin of such a cellular area one may by careful scrutiny observe an extensive alteration from round to stellate types, some cells passing rapidly to an approximate spindle form. The type of motion exhibited by the round cells, when observable, is well shown by fig. 4, cells *a–c* being observed at the extreme margin of the round cell area, cells *d–e* just within the margin, and cells *f–g* well in the interior of the area.

While the general trend of cell change is from round to stellate to spindle cells, a change may often be observed to occur in the reverse direction, as occurred to the cell shown in fig. 5, and that in fig. 6. Such retrograde changes are less frequently observed, and the transformation is less extensive than are the progressive changes from the round to the stellate forms. The retrograde stellate phase is also more frequently of a transient character (fig. 5). Thus, a stellate cell may by retraction of its processes temporarily assume a spheroidal form but it soon again projects pseudopods and regains its stellate character. Or a typical, bipolar, spindle shaped cell may extend a third process, or even several additional processes (figs. 2, 5 and 6), but, so far as I have

observed, such processes are limited in size and usually of short duration. This reverse transformation may be likened to an elastic rebound brought about by an inherent resistance to change of form reacting against an impelling force which directs the transformation from the round to the spindle type. The cell frequently balks at the change, but the general trend from round to stellate and from stellate to spindle form is inevitable.

Motion resulting in change of form is perhaps most active in the stellate type of connective tissue cell. The general trend of this motion seems to be indicated in fig. 5 *I*, in which a typical round cell selected for observation at the margin of the round cell mass in a pectoral fin of a 6 mm. embryo was seen within a period of six minutes to elongate and then to pass through successive stellate shapes to a typical spindle form. But the succession is not always so rapid. Stellate cells exhibit all sorts of morphological transformations in rapid sequence (fig. 7) and this stage of connective tissue development is of relatively more transient duration than either the preceding or the succeeding stage. Moreover, the shape of the cell is undoubtedly influenced to some extent by its surroundings and the duration of a particular stellate, spindle or lamellar shape may in some cases be thus determined.

Likewise, spindle cells undergo considerable transformations in form, the most frequent of which undoubtedly result in the lamellar shapes on the one and in the stellate on the other hand. Because of the limitations of the microscope in the delineation of the 'third dimension' it is most difficult in the colorless living tissues to differentiate between the lamellar and spindle types of cell but the evidence of fixed and stained tissues shows the lamellar to be the more mature, the spindle the earlier type, and I have observed nothing in the living tissues to indicate the contrary unless indeed it be that both types appear to be somewhat dependent on their surroundings, for as already stated these forms, in the same cell, seem to be more or less interchangeable. That spindle cells frequently and freely revert to the stellate type there is abundant evidence. There is also evidence that these cells may be capable of still further transformations than those of mere form. A syncytial stage in the development of connective tissue has

long been assumed. That this stage in its most typical form presents those cell pictures which we are accustomed to regard as stellate cells is well known. It is generally recognized that this syncytial stage passes into one in which the fibers appear and the syncytium is replaced by a tissue of cells and fibers. The syncytial stage has been presumed to be preceded by a cellular stage and to those who have traced the origin of the mesoderm from the time of egg fertilization it would appear logical, even necessary, that at a sufficiently early period a cellular character must obtain, though Mall has questioned the preëxistence of this cellular condition. The transformation from the cellular to the syncytial condition has been ascribed on the basis of stained sections, to either of two processes: either the syncytium arises by incomplete division of preëxisting cells or the syncytium results from the fusion of the preëxisting cells. That some syncytia arise by incomplete cell division is very probably true. This appears specially obvious in such placental tissues as the superficial cells of the chorionic villi. I know of no convincing evidence that it does occur in the connective tissues.

Since I have been unable to observe mitotic figures in the living connective tissue cells of the fish which are under discussion I cannot offer any evidence pro or con the origin of a connective tissue syncytium by incomplete cell division. I have, however, frequently observed a phenomenon which simulates the fusion of processes of adjacent stellate cells after the manner of a typical connective tissue syncytium. In figs. 2 and 8 *II* the neighboring cells, which were at first entirely distinct and separate, were within a brief period seen to send out processes which on contact apparently fused. But of course one cannot say without subsequent fixation and staining of the identical cells, a process presenting the greatest difficulties, that the fusion was actual and complete. Even in stained sections the question is often difficult to determine. While the fusion was apparent I am not at all sure that it was actual. Not, however, in every case when cell came into contact with cell did such apparent fusion occur. This is shown in fig. 8 *I*, in which processes from the cells *a* and *b* came into contact tip to tip, yet though fusion seemed imminent it did not occur

and the contour of each cell at the point of contact remained clear and distinct. Moreover it would seem that since connective tissue cells move extensively along the surfaces of the syncytium that syncytium could scarcely arise by fusion of its cells.

The spindle cells exhibit a certain stereotropism. They are prone to take their position alongside a connective tissue fiber or fiber bundle or against the surface of a blood-vessel or dermal fin-ray. When in contact with a broad surface, such as that of a blood-vessel or one of the lepidotrichia, the cells frequently assume a flattened, lamellar form. This is shown by stained sections, in which that type of cell predominates in these locations, and by the observation of living spindle cells which frequently move up to a blood-vessel or a fin-ray and then become so thinned out against the surface that they finally vanish, being in the living tissue indistinguishable from the refraction lines which surround the larger bodies. Again, the spindle cells very frequently move up to a connective tissue fiber or bundle and then elongate along the narrow filament until, as before, the cell finally appears to vanish by its extreme attenuation. Such a result was observed a moment later than the recorded observation in the case of the cell shown in fig. 5*I*.

It frequently happens that the spindle cells after such elongation again thicken to a typical spindle form, and may even throw out other processes, but in so doing, if the cell is observed in relation to some relatively fixed point, e.g., a joint of a dermal fin-ray, a chromatophore, or a blood-vessel, it will be seen that the cell has changed its relative position; it has exhibited locomotion. Locomotion is not a distinguishing character of the spindle cell; it is exhibited by the stellate cells, possibly also to a very limited extent by those round cells which are only just beginning to present pseudopod formation. But the character of the locomotion in the several types of cells differs decidedly. In the stellate type locomotion may take any direction and resembles a very active amoeboid motion, processes being extended along the surfaces of fine fibers, then either retracted or increased in size until the whole cell has come to occupy the place of the former process. Though locomotion in the stellate cells is not entirely confined

to the direction of visible fiber lines, yet a projecting process of such a cell often appears to envelope or to become coincident with a fiber. In the spindle cells locomotion is always so far as I have observed, in the direction of the fiber lines: usually these cells merely slide along the surface of fibers, blood-vessels and similar structures.

I have observed that the stellate cells are more prone to lie in relation with the finer, the spindle cells with the coarser fibers; the coarser fibers in most cases, because of their size, being presumably fiber bundles rather than single fibers. This relationship is to be expected in as much as in stained preparations one finds the stellate cells present with those finer fibers which represent the earlier stages in fiber formation.

That fibers do lie without the cell in both embryonic and mature connective tissue is generally conceded. That they lie within the cell in reticular tissue, which in a way is comparable to an early or embryonic type of connective tissue, I have recently demonstrated by means of the Bielschowsky stain.[1] The types of fiber development by fusion of intracellular granules described by Spuler and by Lavini though perhaps not conclusively demonstrated, at least show that certain granules which are in relation with the first appearance of fibrils do lie within the substance of the stellate, mesodermal, connective tissue cells. Moreover, I have found in embryonic tissues (fig. 9) just such appearances as I have described for reticular tissue.[2] By means of the Bielschowsky method such appearances can be shown throughout embryonic connective tissue. I have observed them in pig embryos, of various ages, in the limb buds, the head, the cervical region, and in the back throughout the whole length of the embryo from the occiput to the caudal tip, also in the umbilical cord. In many of these locations I have made similar observations on human embryos of older stages but in which the connective tissue was still actively developing. One is at a loss to explain the method by which fibers arising within the cells arrive at a location outside the cell body when these cells are in active motion. The

[1] Am. Jour. Anat., vol. 13, page 277, 1911.

[2] Loc. cit., in which see especially fig. 4 and fig. 8, pages 285 and 289.

ectoplasmic theory of Hansen does not satisfactorily account for it and its elaboration by Mall is not as specific in this particular as one might wish. These theories do not appear to fully harmonize with the relatively active motion and locomotion of the connective tissue cells which I have observed in the fins of living fish and which Harrison, Burrows and others have also to some extent recognized in tissue cultures. The cells are not sufficiently quiescent to permit of endoplasmic retraction with deposit of ectoplasmic fibers unless this retraction is rapidly performed, in which case it should be observable in the living embryo. I have in one or two cases suspected such a method of deposit but have not as yet been able to convince myself that it actually occurs; in fact I now doubt if it occurs at all.

The ectoplasmic theory presupposes that the fibers arise at the surface of the cell. This I have found to be not always the case. The clear delineation of fibers by the Bielschowsky method makes it possible to follow their course within the cell more carefully than ever before and I find that the blackened fibrils within the cell both in pig embryos (fig. 9) and in the fish's fin very frequently pass close to the nucleus, sometimes ending almost in contact with this structure, but more frequently passing by so closely as to be in actual contact with the nuclear membrane. I am aware that Golowinski using the iron haematoxylin method, demonstrated the presence of fibers at the surface of the connective tissue cells of the umbilical cord and that the apparent relation to the nucleus was explained by him as due to obliquity of section. But I have not in my preparations been able to convince myself of the adequacy of this explanation. I have found fibers to be not always at the surface of the cell, they may and frequently do penetrate entirely through the cytoplasm of the cell, as I have previously described[3] for mature reticular tissue. In the developing connective tissue, as well as in reticular tissue, such penetration of cells by the fibers is so frequent as to appear quite characteristic. It seems to me that the intimate relation of connective tissue cells and fibers in embryonic tissues can only be accounted for by tak-

[3] Loc. cit., see fig. 10, page 293.

ing cognizance of the plasticity of the connective tissue cellular cytoplasm, and also of the active motion of connective tissue cells during the period in which the fibers are being formed, so that the finer connective tissue fibers become, by the cellular activity, embedded in the plastic cytoplasm of the cells during their stereotropic locomotion. The plastic character of the cellular cytoplasm is admirably shown by the rapid changes in form of the connective tissue cells in the fins of living fish embryos.

I have already stated that the spindle cells of connective tissue in the fins of living fish undergo active locomotion. In fact this seems to be a most prominent function of the spindle cell type. Most frequently the cell glides along connective tissue fibers which often appear to be thus partially enveloped by the cytoplasm. In recording this stereotropism I am able to corroborate, for the living cells of embryo fish, the observations of Harrison on tissue cultures in which he finds that the connective tissue cells are specially prone to follow along the surface of fixed objects. Such objects in normal living subjects are most frequently the connective tissue fibers and fiber-bundles already deposited, though as previously stated, I have also observed connective tissue cells moving along the surface of the dermal fin rays and of blood-vessels. In this form of activity the cells adapt themselves more or less to the shape of the surface along which they are moving. They wrap themselves about or rotate around the finer fibers (fig. 3) and they flatten themselves against the larger objects (figs. 2, 3 and 5). In this attenuated condition they still move along the surface of fibers, often at a considerable rate of speed. One such cell I have recorded in a preliminary communication[4] was found in ten minutes to have covered a distance of 50μ, a rate of 1μ in every twelve seconds.

The striking similarity of the living, spindle, connective tissue cells to those of fixed tissue is indicated in fig. 10 which shows several such cells from a 100 mm. pig embryo. The magnification is the same as that used for the observation of the living cells. The similarity in the form of the cell and the relation of cells to fibers is apparent on comparison with the preceding figures. One

[4] Biol. Bull., vol. 21, page 272, fig. 2, 1911.

can scarcely avoid the interpretation that the cells shown in fig. 10 were at the moment of 'fixation' moving along the surface of the fiber bundles against which they lie.

The pronounced morphological relation between the connective tissue cells and fibers cannot but have an equally close functional relation. What those functional relations may be we are not now possessed of the data to fully determine.

Further studies will be necessary to fully understand the part played by the active moving connective tissue cells in the production and growth of the collaginous fibers. So far as they are now determined the essential phases of the process in which the connective tissue cells are concerned appear to be: (1) the connective tissue arises from a primitive mass of round mesenchymal cells; (2) there is a change of form from round to stellate, to spindle, and eventually to lamellar cells; (3) certain fibers seem first to appear within the cells, possibly at their surface (Hansen, Golowinski); (4) there is formed a reticulum pervading the intercellular ground substance whose fibers may be, though they not necessarily are, identical with those first arising within the cell; (5) coincident with the origin of fibers there begin amoeboid movements in the stellate and spindle cells; (6) there is an increase in size and number of fibers in the reticulum and they aggregate into bundles, synchronously with which first the stellate and later the spindle cells move along the surface of the fiber and fiber bundles.

In conclusion I desire to express my sincere thanks to the Marine Biological Laboratory at Woods Hole, Massachusetts, for the opportunities so kindly placed at my disposal.

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PLATE 1

EXPLANATION OF FIGURES

1 From a Fundulus embryo, of 5.5 mm. total length, showing beginning amoeboid movements of two cells, *a* and *b*, on the border of the round cell area at the posterior extremity of the ventral median fin. The observation extends over a period of fifteen minutes. The last seven drawings were made without change of focus for the purpose of eliminating variation in form due to the examination of different levels. *a*, *b*, two connective tissue cells. *Ch*, prominence on the surface of a chromatophore, the body of the cell is not represented. The numerals in this and succeeding figures indicate the exact time at which each recorded drawing was completed.

2 Connective tissue cells, one of which, *a*, exhibits transformation from a spindle to a stellate type, and another, *b*, becomes flattened against a connective tissue fiber. There was an apparent anastomosis between cell *a* and the protoplasmic process *p* of an adjacent cell. *f*, connective tissue fiber; *j*, margin of a joint of a fin ray, giving a fixed point in relation to which locomotion may be determined. Other letters and numerals as in the preceding figures.

3 Two connective tissue cells exhibiting some locomotion. One of these, *a*, assumed a lamellar like relation to a fiber bundle while rotating about it. At 9.25 A.M. this cell became momentarily so thin as to almost escape observation. *Ch*, *Ch'*, chromatophores. Other letters and numerals as in the preceding figures.

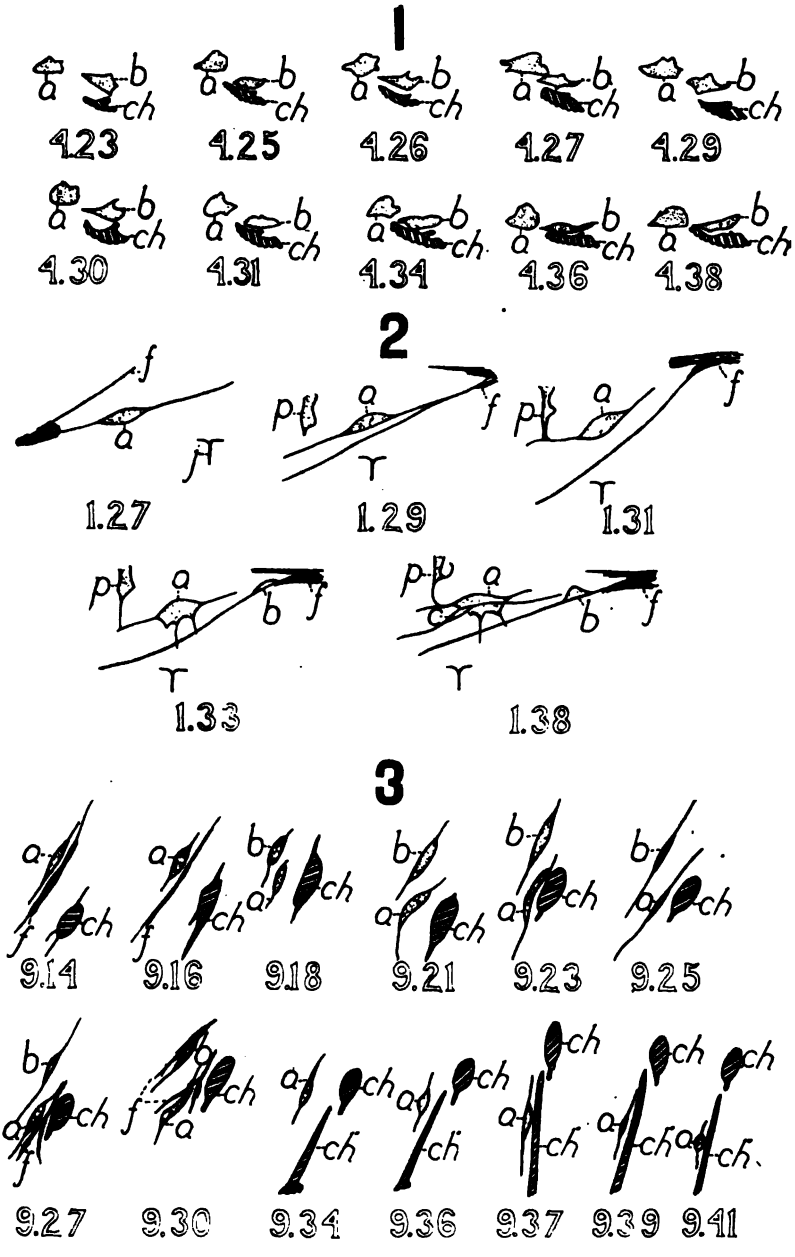


PLATE 2

EXPLANATION OF FIGURES

4 Amoeboid motion resulting in change of form exhibited by connective tissue cells of the primitive or 'round' type. *I*, cells *a-c*, from the extreme margin; *II*, cells *d–e*, from just within the margin; and *III*, cells *f–g*, from the interior of a round cell area. *I* and *II* from the pectoral, *III* from the caudal fin. Numerals as in the preceding figures.

5 *I*, transformation of a round to a spindle cell in the pectoral fin of a Fundulus embryo 6 mm. long, 20 days after fertilization, 11 days after hatching. From 4.20 to 4.22 P.M. there was in this cell an apparently retrograde change from spindle to stellate form but at 4.24 P.M. this had been proven temporary. *II*, transition of a stellate cell to a temporary spindle form. Letters and numerals as in preceding figures.

6 Apparent retrograde change from spindle to a stellate form in a cell undergoing rather slow locomotion. The stellate phase of such cells is nearly always temporary. From the same embryo as fig. 5; *cap.*, blood-capillary. Other letters and numerals as in the preceding figures.

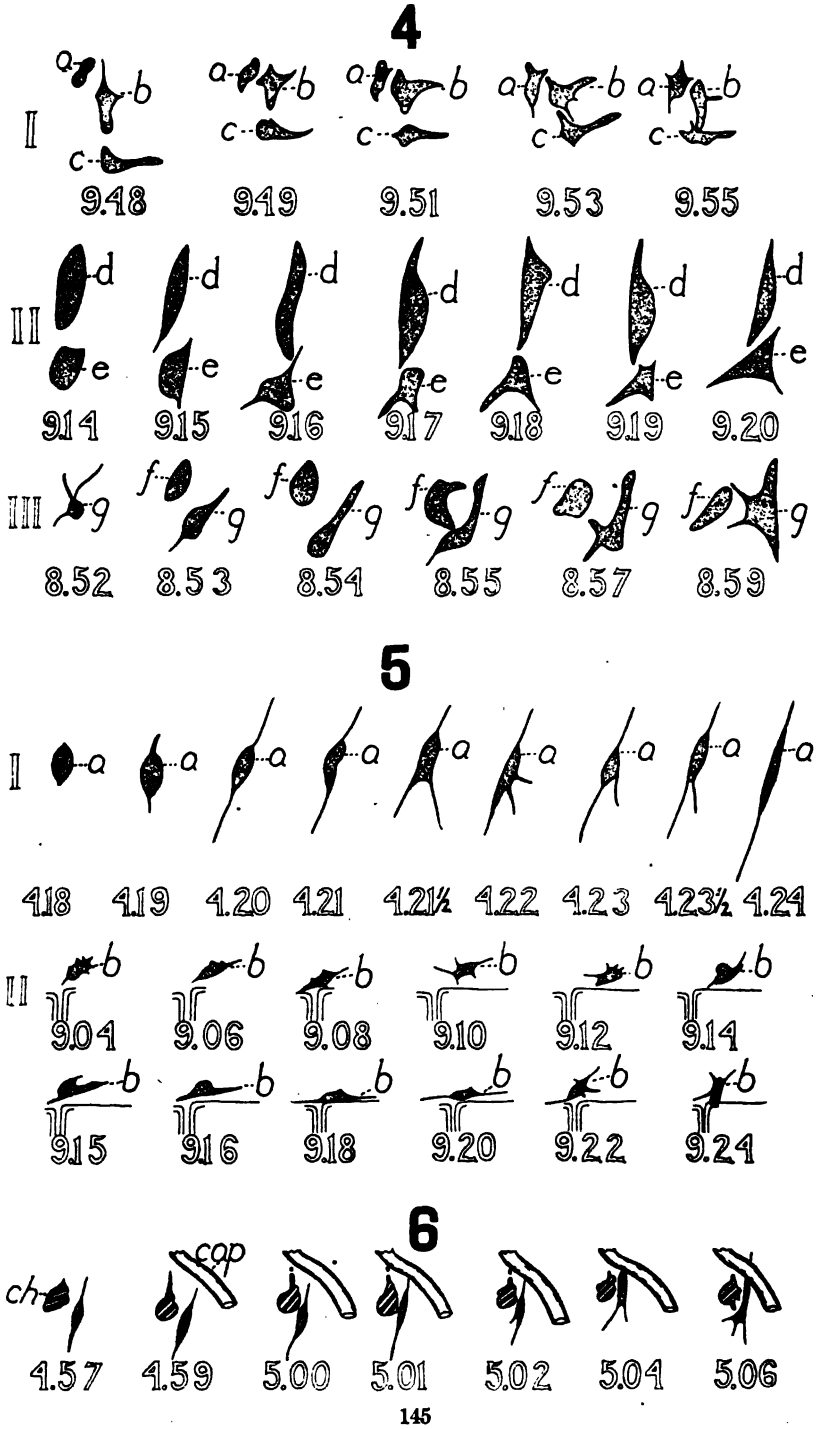


PLATE 3

EXPLANATION OF FIGURE

7 Stellate connective tissue cells from the fins of four embryo fish, *I–IV*, exhibiting rapid change of form. Owing to difficulties of observation it is not possible to make drawings oftener than at 1 to 3 minute intervals; hence, the actual changes of form were much more frequent than the record shows. *A-K*. nine connective tissue cells. Numerals as in the preceding figures.

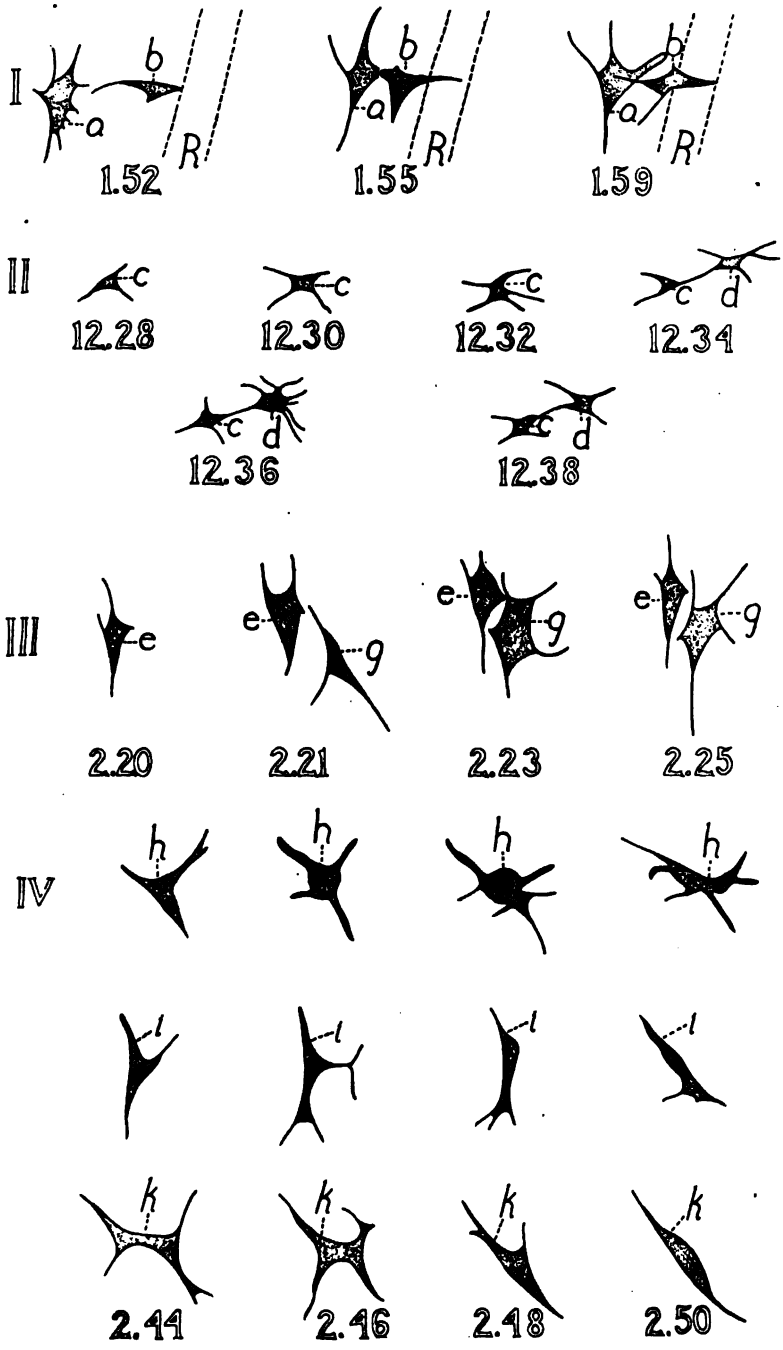


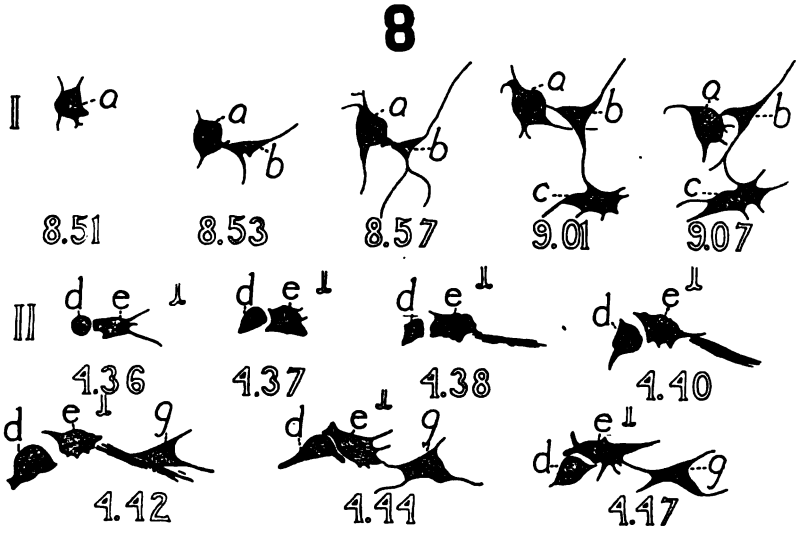
PLATE 4

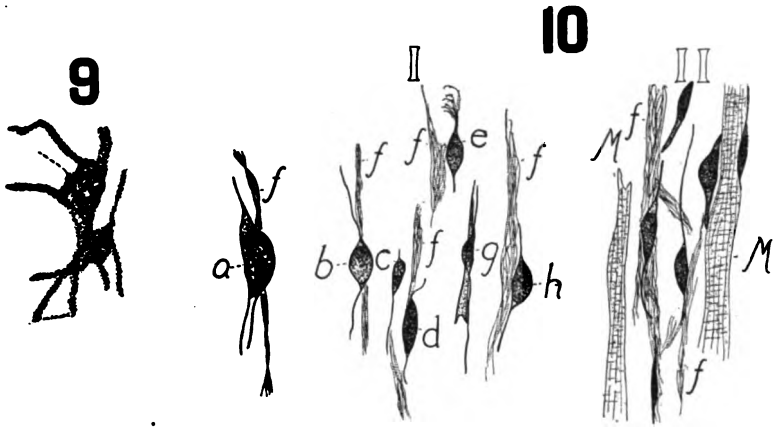
EXPLANATION OF FIGURES

8 Stellate connective tissue cells exhibiting locomotion and, on contact, apparent fusion. *I*, from the pectoral fin of a 10 mm. Fundulus embryo. The fusion between cells *a* and *b* is apparent only. *b* and *c* appear to form part of the anastomosing syncytium. *II*, from the caudal fin of a 6 mm. Fundulus embryo. Cells *e* and *g* on coming into contact at 4.44 P.M. apparently fused after the manner of the cells which form the delicate early connective tissue syncytium. Letters and numerals as in the preceding figures.

9 Stellate connective tissue cells in the subectodermal mesenchymal syncytium of a 25 mm. embryo pig. Fibrils pass through the cells very close to the nucleus. Bielschowsky stain.

10 Connective tissue cells from the praevertebral (*I*) and intermuscular (*II*) connective tissue of a 100 mm. pig embryo. The form of the cells and their contact relations to adjacent connective tissue and muscle fibers is strikingly similar to the amoeboid connective tissue cells of the living fish embryo. The appearance suggests that such cells were quite probably moving along the surfaces with which they are in contact.





The Relation of Muscle Cell to Muscle Fibre in Voluntary striped Muscle.

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(From the Biological Laboratory at Bonn.)

With table VII and VIII.

(Der Redaktion zugegangen am 19. Juli 1912.)

It may be stated in general terms that our present conception of a voluntary muscle fibre is that of an enormously large, multinucleated cell invested by a cell membrane or sarcolemma and containing the several elements of contractility. Or, as a recent textbook of histology phrases it, „Die quergestreifte Muskelfaser wäre also als ein vielkerniges, fadenförmiges Plasmodium mit deutlicher Zellmembran zu bezeichnen" (Sobotta). That this "cell membrane" invests, in addition to the muscle fibrillae and muscle nuclei, at least two entirely different forms of protoplasm,[1]) so far as their morphological appearances are concerned, there has been but little question but at the same time comparatively little mention in the literature of this feature. The one form immediately invests the nuclei and possesses the characteristics of cellular protoplasm as elsewhere observed; a granule-laden spongioplasm network with interstices of clear hyaloplasm. These general features are wanting in the other form, i. e., the sarcoplasm of the muscle fibre.

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[1]) Many of the published plates of such investigators as Heidenhain, Holmgren, Nussbaum, Schneider, Marceau, Werner, Prenant, etc. represent these differences most clearly.

These remarks, naturally, apply more particularly to the adult fibre. In the developing fibres the quantity of protoplasm investing the nuclei is relatively greater and much more coarsely granular. During this period it possesses apparently very intimate developmental relations to the muscle fibrillae and to the sarcoplasm since its quantity diminishes as the latter increases.

Investigators who have worked upon the structure of striped muscle have in general regarded a close functional relationship as existing between the sarcoplasm and muscle fibrillae on the one hand and between the muscle nuclei and adjacent differentiated protoplasm on the other, yet the exact morphological connection existing between the latter and the sarcoplasm has been but little examined beyond the general assumption that the two stood in intimate structural contiguity with each other.

It was with the express intention of carefully considering this relationship that I undertook the study of the muscle fibre, confining my efforts for the present to the voluntary cross-striated muscles of the tadpole, frog, chicken, calf, white mouse, gray mouse, and cat. I used for the investigation specimens of the extrinsic muscles of the eyeball, intercostales, rectus abdominalis, latissimus dorsi, adductors of the thigh, sartorius, flexors of the foot together with the caudal muscles of the tadpole. These various muscles were fixed in sublimate, FLEMMING's solution, MEVES's solution, and in 96 % alcohol. They were imbedded in paraffin, some after SCHULTZE's collodion and cedar oil method, and sectioned longitudinally, obliquely, and transversely in thicknesses varying from 2 to 5 μ. As stains I made use of picric acid alcohol, fuchsin S, picro-fuchsin, GAGE's chloral-hematoxylin, SCHULTZE's alcoholic-hematoxylin, eosin-xylene, and an alkaline ferric-tannate solution.

The sketches which I present, while they were made from actual specimens, are accurate, however, only so far as concerns the exact relationship of the protoplasm immediately investing the muscle nuclei to the muscle fibrillae and to the sarcoplasm of the muscle fibre. That is to say, inasmuch as the structural features of these other muscle elements did not bear directly upon the subject of the investigation, I made no effort, beyond a mere approximation of accuracy, to delineate either their structure and their relationship to each other or the ultimate structural features of the muscle nuclei. Accordingly, these figures are only to be regarded as authoritative with these two qualifications.

In most instances, I have represented only a single muscle nucleus with the adjacent muscle fibrillae and sarcoplasm. The relations shown were found to be characteristically present throughout the entire series. For the demonstration of the facts, however, I deemed it sufficient to represent only a single nucleus and the immediately adjacent muscle fibrillae. Hence these are not to be regarded as exceptional instances.

I have represented in Fig. I a portion of a fibre of an extrinsic muscle of the eyeball of a white mouse fourteen days old. At this age the eyelids are open. The fibre is invested by its sarcolemma lying upon which at one end there are several connective tissue cells and fibres. In the middle of the muscle fibre a nucleus surrounded by a considerable amount of protoplasm is shown. The characteristic features of cellular protoplasm as generally recognized are to be observed in connection with this protoplasm, i. e., strands of granule-laden spongioplasm composing a network which encloses clear hyaloplasm. The exact extent of this protoplasm in either direction is not shown, because of its passage out of the level of the oblique section. It is to be observed, however, that the structure of this protoplasm presents a marked contrast to that of the relatively uniformly and faintly staining sarcoplasm of the muscle fibre. It is further to be noted that the latter is sharply delimited from the spongioplasm network by the presence of a distinct membrane. The fibrils of spongioplasm can be observed to be attached to the internal surface of this membrane. That the existence of this membrane, as a distinct structural entity limiting the protoplasm, is not merely an appearance owing to the presence of overlying contingent muscle fibrillae is most clearly demonstrated by reason of the obliquity of the section. The relatively clear spaces intervening between the obliquely-sectioned fibrillae are observed to be bridged over by this structure. This particular feature is more clearly represented in the lower end of the section. In brief, then, we are dealing here with features which represent every characteristic of cell structure, a nucleus, cell protoplasm, with spongioplasm and hyaloplasm, and a cell membrane, all imbedded in a voluntary muscle fibre.

The clear spaces observed within the cell body immediately above and below the level of the nucleus I have regarded as artefactitious, referable as much to a retraction of the nucleus as to a corresponding shrinkage of the cellular protoplasm. At the same time attention must be called to the fact that in spite of these

circumstances there is no indication in the section whatever either of a retraction of the spongioplasm from the cell wall or of the cell wall from the sarcoplasm outlying it. In many of the specimens, I have identified spongioplasm fibrillae which were attached to the nuclear membrane by one end and by the other to the cell wall.

I desire particularly to call attention to the relation of the cell wall to the nuclear membrane at the level of the nucleus. The two lie so close together as to be apparently fused. It is owing to the juxtaposition of these two structures that, when a muscle fibre presenting such a cell is studied in cross-section at the level of the nucleus, the cell membrane escapes observation altogether and the nucleus appears to lie imbedded in and in direct contact with the sarcoplasm. My own transverse sections of this same muscle and of all of the other muscles which I have studied, furnish ample evidence of this appearence, yet in those transverse sections in which, either as the result of the agents used in the preparation of the specimen or where, as the result of a tear in the muscle fibre, the nucleus was pulled away from the sarcoplasm, the presence of the cell membrane was then manifested and its existence at the level of the nucleus established. Furthermore, when transections which exhibit no traces whatever of shrinkage are studied either directly above or immediately below the level of the nucleus, the cell membrane can be seen most clearly and the fact observed, as well that it passes completely and uninterruptedly around the cell protoplasm.

In fig. II I have represented an oblique section of a similar muscle fibre presenting appearances comparable to those just described. A muscle cell occupies the middle of the fibre. Several connective tissue cells and fibrillae lie upon the sarcolemma. Very often in studying fibres of the extrinsic eye muscles of the white mouse, I have encountered specimens in which, apart from the characteristic muscle spindles, several muscle nuclei were closely associated with each other in the middle of the fibre. I have found as many as five nuclei so situated, imbedded in a protoplasmic mass which extended in the long axis of the fibre. This mass was everywhere marked off from the sarcoplasm by a membrane, similar in morphological structure and in relations to that which I have described above in connection with fig. I.

The question naturally arising in such instances was whether such a protoplasmic mass represented a single muscle cell containing several nuclei or whether the nuclei each represented distinct cells

whose boundaries, because of their thinness, were not to be separately differentiated. In most instances I was not able to answer this question even in specimens studied under the best conditions of fixation and of stain. The specimen at hand furnishes, however, a suggestion of the probable solution of the difficulty. I can commit myself, however, to a positive statement regarding the general problem only so far as this one cell in particular is concerned. The protoplasm occupying the upper portion of the sketch and investing the nucleus there situated, is marked off from that found in the lower end by an obliquely-placed membrane, which, though faintly stained and somewhat indistinct, has the morphological appearance of a true cell wall. The lower mass of protoplasm contains as the next section in the series shows, also a nucleus.

The presence of such elongated masses of protoplasm is not restricted to the mouse. They can be observed as well in the extrinsic eye muscles of the three-weeks chicken. As yet, however, I have not detected these masses in the adult muscles of the thigh, leg, abdomen, or thorax of the white mouse, frog, chicken, gray mouse, or cat. In such muscles the nuclei are usually isolated from each other, while the quantity of protoplasm immediately investing them is relatively much less in amount.

Figure III represents a portion of a thigh muscle of an adult frog, showing features similar to those observed in connection with the white mouse muscles. The section was cut obliquely; consequently the muscle fibrillae cannot be traced for any considerable distance. I have represented in the sketch a single muscle cell with the adjacent fibrillae. This cell was imbedded in the muscle fibre and bore no immediate structural relationship to the sarcolemma. By reason of the obliquity of the section the internal surface of the cell membrane can be seen at the upper end of the cell. There is no question as to its presence or as to its continuity. As a distinct limiting membrane it can be traced from the vicinity of the fibrillae situated upon one side of the cell to those on the other.

Very often it is difficult to differentiate between the telophragma (Z) lines of the muscle fibrillae and the reflected cell membrane at the poles of the cell. In this particular specimen, however, this difficulty is lessened by reason of the fact that the features of cross-striation of the muscle are not distinctly marked. The cell wall, accordingly, can be seen all the more clearly. By reason also of the alcoholic hematoxylin stain used on this prepa-

ration the fibrillae are much lighter in color than the cell wall. Hence the possibility in a longitudinal section of mistaking the border of a muscle fibril for the cut edge of the cell wall is also eliminated. The cell membrane is readily demonstrable as a formed structure separated from the muscle fibrillae by a narrow interval of sarcoplasm. What is, however, a much more significant fact pointing towards the presence of the cell wall is, as the sketch shows, the presence of several strands of spongioplasm which terminate upon this cell wall. In no cell which I have studied have I ever seen a fibril of spongioplasm terminating upon a muscle fibril.

At the lower end of the cell two, sectioned, muscle fibrillae lie upon the external surface of the cell wall. These fibrillae passed out of the plane of the section in their passage around the cell body. Several published plates would lead us to infer that in some instances at least, muscle fibrillae terminated at a muscle cell. I have never observed such an instance, however, in my own specimens. In every section the fibrillae diverged from each other in order to pass uninterruptedly beyond the cell.

The remarks, which I made regarding the relation of the cell wall to the nuclear membrane opposite the center of the nucleus in connection with the mouse muscles, are to be emphasized at this place with regard to the frog muscle. The cell membrane everywhere separates the nuclear membrane from the sarcoplasm. And, likewise, in those specimens, where a shrinkage of the nucleus has taken place, its presence is most readily made out.

In some of the transverse sections of frog muscle the muscle fibrillae were torn away from each other. I have represented such a section in fig. IV. The sketch was taken at an optical level a little above the center of the nucleus. This shows a cell located in the middle of a muscle fibre. The muscle fibrillae lying above and to the left were undoubtedly torn away from a more intimate relation with the cell. From a careful study of the preparation, I concluded that the nucleus as well had been removed slightly in a direction upwards and to the left. In this direction the cell wall itself was torn. It remained intact, however, and in its proper position relative to the muscle fibrillae and sarcoplasm upon the other two sides. At the left side of the cell the edge of the cell wall can be seen to project from its attached position to the sarcoplasm into the clear space occasioned by the tear in the muscle fibre. It is further to be observed that the part projecting into

this tear is unaccompanied by sarcoplasm. These features are less clearly outlined upon the right side of the cell. The specimen furnishes to my mind the most conclusive evidence as to the existence of the cell membrane as a distinct, independent, morphological structure.

A cell membrane is characteristically associated not only with those muscle nuclei which are located in the middle of a muscle fibre, but as well with peripheral-lying nuclei. I have observed the same relationship of nucleus to protoplasm, of protoplasm to cell wall, and of cell wall to sarcoplasm in connection with those nuclei which are applied to the sarcolemma. The cell membrane everywhere separates the sarcoplasm from the spongioplasm network of the cell. Upon that surface of the cell which is directly applied to the perimysium, however, the cell wall fuses with that structure and loses its identity.

In figs. V and VI are represented portions of muscle fibres removed from the thigh of an adult cat. Both of these cells lay upon the periphery of the muscle fibre in contact with the perimysium. The fibre in fig. V was sectioned longitudinally and that in fig. VI obliquely. A fold occurring at the level of the nucleus accounts for the transected fibrillae in the first. In both instances the cell membrane can be seen distinctly. It separates the cellular protoplasm containing the nucleus from the contractile elements of the muscle fibre. Upon that side of the cell which is applied to the perimysium it is fused with this structure and cannot be traced.

In the study of similar preparations presenting these features it was necessary to differentiate muscle nuclei applied directly to the internal surface of the perimysium from those connective tissue cells lying upon the external surface of that structure and from those which were, to all appearances, in the perimysium itself.[1] The fact is quite self-evident in the figures, particularly in fig. VI, that the nucleus is imbedded among the muscle fibrillae. That is to say, this relation is more intimate than would be expected were the cell situated outside of the sarcolemma but causing an indentation

[1] This last statement requires some qualifications in view of the recent work of Schieffferdecker upon the sarcolemma. The nuclei to which I refer are so situated within what appears to be the sarcolemma that this latter structure splits apparently to enclose them. At any rate it is not possible to see any formed structure between such nuclei and the relatively clear sarcoplasm. I will comment more at length upon these special features, however, in a forth-coming paper.

of the muscle fibre. Furthermore, the morphological appearances of these cell walls are precisely similar to those noted before as characteristic of cells found imbedded among the fibrillae in the middle of a muscle fibre. Again, a difference can very readily be observed between these peripheral muscle cells and what, to all appearances, are cells located in the perimysium. The latter are small and spindle-shaped, with a compact and deeply-staining nucleus surrounded by a relatively small amount of dense and uniformly deeply-stained protoplasm.

From the study of extrinsic muscles of the eyeball of a calf, I was convinced of the presence of a cell membrane similar in relations and in its morphology to that which I had observed in the other vertebrates.

In fig. VII I have represented an entire muscle fibre cut transversely. The sketch was taken a little above the level of the center of the nucleus. At the latter level the cell membrane was not visible as a separate structure owing, as I have previously mentioned, to its contact with the nuclear outline. At the level represented, however, it is readily seen. I desire to direct particular attention to its relation to the muscle fibrillae. I have never found it in direct contact with these structures. An interval of sarcoplasm always intervenes between the two. In general terms it may be stated that this intervening layer of sarcoplasm averages in thickness to the cross diameter of a muscle fibril.

The sections of latissimus dorsi of the three-week-old chicken also show the same general features of cell protoplasm and cell wall. In fig. VIII I have represented a transection of an entire muscle fibre under the same conditions as those in fig. VII. The cell wall lies at a little distance from the adjacent fibrillae. It stains very deeply in contrast to the intervening sarcoplasm. At levels above and below that of the nucleus fibrils of spongioplasm can be traced to its internal surface where they find an attachment.

In the study of the longitudinal preparations of striped muscle fibres, there are introduced several factors which render the identification of the wall of the muscle cells particularly difficult. First among these to be mentioned, is the presence of the parallel-running muscle fibrillae. The free edge of a fibril may be very easily mistaken for the cell membrane. What is more significant, however, is the overlying or underlying of the membrane by these fibrillae thereby overshadowing the delicate cell outline. Still another difficulty is encountered at the extremities of the cells where the telo-

phragma lines bridge over the narrowed cell protoplasm. Dependent upon this latter factor is the difficulty in the determination of the cell extent particularly enhanced. Then, lastly, scattered among the muscle fibres many connective tissue cells are found whose long axis corresponds very often to that of the muscle cells and, accordingly, to that of the muscle fibre. In their general outline, in the morphological characteristics of their protoplasm, and in that of their nucleus, as well, these cells often resemble the muscle cells very closely. Therefore, in the instance of such cells occurring upon those aspects of the muscle fibre either facing towards, or turned away from the observer it is, at times, not easy to differentiate the cells as muscle cells or as connective tissue cells. In other words to say whether the cells lie inside of the sarcolemma or outside of it.

In fig. IX I have represented a portion of a fibre from an extrinsic muscle of the eyeball of a calf. Two muscle cells, whose nuclei indent the longitudinally-cut fibre, are to be seen. The marked morphological differences between the protoplasm of these cells and the sarcoplasm of the fibre are most apparent. Notwithstanding, this fact by reason of the presence of the parallel muscle fibrillae, it is exceedingly difficult in this specimen to determine the exact limits of this protoplasm. In specimens stained with alcoholic hematoxylin and then properly extracted, however, the differentiation of cell membrane from fibrillae is rendered possible. Serving as good controls for such longitudinal sections are obliquely-sectioned preparations, for in such the cell wall can most readily be distinguished apart from the fibrillae.

In focusing down through this particular specimen, I encountered, first, the telophragma lines with the muscle fibrillae upon the upper aspect of the fibre, then the cell nucleus and protoplasm, and lastly, the same lines upon the under aspect of the fibre. Hence I was most positive that these were muscle cells within the fibre and not connective tissue cells either overlying or underlying the muscle fibre border.[1])

At this place again, I desire to point out the closeness of approximation of the nuclei to the muscle fibrillae. The latter are

[1]) I have projected the telophragma lines upon the same plane with the nucleus and cell protoplasm. They do not encircle the peripheral cells, however, nor do they traverse the cell protoplasm. I have made these points the subject of a forth-coming paper on the sarcolemma.

diverted out of their course slightly at its level. At this same level the cell wall and nuclear membrane are in contact with each other and are not separately distinguishable.

The structural relationships noted in connection with the calf's-eye muscle, I observed as well in the latissimus dorsi muscle of the three weeks chicken. Fig. X represents a portion of a fibre from this muscle. This is an alcoholic hematoxylin preparation which, notwithstanding the proximity of the muscle fibrillae, demonstrates the cell outlines very clearly. That the two cells seen were muscle cells and not connective-tissue cells located without the perimysium, I established by determining their position relative to the telophragma lines.

A muscle fibre from an extrinsic muscle of the eyeball of a white mouse is represented in fig. XI. This is a chloral-hematoxylin preparation. The outlines of the two cells upon the right of the figure are not clearly seen. Both of these cells, however, are muscle cells. In the instance of the single cell situated upon the left border of the fibre, we can note a morphological similarity to those represented in fig. IX.

In the specimens of leg, intercostal, and rectus abdominalis muscles of the adult mouse, the muscle cells are relatively less numerous, yet the cell membrane is everywhere clearly defined.

I present fig. XII for the purpose of demontrating one of the difficulties encountered in establishing the extent of the protoplasm of a cell in a longitudinal direction. The specimen is a tail muscle of a tadpole about 5,0 cm long. In either direction, as is shown, the cell protoplasm is drawn out to a pointed extremity lying between parallel-running muscle fibrillae, and occupying a space about equal in cross-diameter to that of the average interval existing between adjacent fibrillae. It is readily understood from a careful study of the figure how the elements of cross-striation, where overlying the narrow, pointed extremity of the cell, are very readily confused with that extremity. Indeed, for this reason, it is extremely difficult to determine in any given instance the true limit of the cell.

In conclusion it may be stated in general terms that the cell membrane is seen to best advantage in those oblique sections which are cut at a thickness of 2 μ and are stained with either alcoholic hematoxylin or with alkaline ferric tannate, and in such instances where the cut edge of the membrane, owing to the obliquity of the receding cell wall, is underlaid by nothing but the clear hyaloplasm of the cell. In many such instances where the staining is of the

proper depth the internal surface of the membrane itself can be made out and its continuity, delicacy, and similarity to other cell walls observed. With the hematoxylin stain, the clear fibrils of spongioplasm can be seen to be attached to its internal surface. The interval of sarcoplasm existing between the muscle fibrillae and the cell membrane can also be noted. That the membrane is more than the thickened edge of the sarcoplasm seems to my mind to be established by its staining reaction, its presence as a distinct lamina separate from the sarcoplasm, in those instances where the muscle fibre has been torn, and by the attachment of fibrils of spongioplasm to its internal surface. That it is not an artefact produced by the shrinkage of protoplasm toward and upon the edge of the sarcoplasm, I believe, because it can be clearly observed in specimens where there is no indication whatever of shrinkage elsewhere in the tissues.

Concerning the query as to whether all of the nuclei with their surrounding protoplasm are invested with a cell wall, I can answer, that in all of the specimens which I have as yet studied and which were properly cut and stained, I have been able to make out the cell wall. Because of this fact, in such muscles as the intercostales which I have studied in their entirety, I consider that the muscle fibrillae are to be regarded as extracellular and not, as we have heretofore believed, intracellular. In the instance of the other voluntary striped muscles, since I have as yet observed no cell which did not possess a definite cell wall, I feel reasonably sure, though I have not as yet sectioned and studied an adult muscle as the sartorius or latissimus dorsi of the cat in its entirety, that the generalization, that the voluntary, striped-muscle fibrillae of the adult should be regarded as extracellular, is justifiable.

The apparent reason why this membrane has been overlooked by the very great number of investigators working upon muscle is referable not so much, I believe, to a fixation method fault as to a staining defect. I have, for instance, restained slides which were at first stained with eosin, fuchsin S, picro-fuchsin, and even with alcoholic hematoxylin, in which, however, the extraction of the stain from the muscle fibrillae was relatively not sufficient, and in which, accordingly, the cell membrane could not be seen, and in the same section after treatment with alcoholic hematoxylin and after a proper extraction of that stain, I have been able to define the membrane most clearly.

In order to guard against the false appearances produced by

shrinkage in stained sections, I studied many preparations of living muscle of the frog and the tadpole. In all of them the muscle cell outlines were very clearly seen. In fig. XIII I have represented a single muscle cell with the adjacent fibrillae from a thigh muscle of an adult frog. The outline of this sketch was made within ten minutes from the time that the portion of muscle was removed from the thigh of the living animal. No fixatives or other chemicals producing shinkage were employed on the specimen. The presumption is fair, I believe, that the sketch represents the condition as seen in a living muscle fibre. The outline of the cell body with its distinct cell wall is most clearly seen. The muscle fibrillae are very lightly stained with dilute methylene blue in comparison to the protoplasm of the cell body. The fibrils of spongioplasm can be observed to be attached to the cell wall. This preparation eliminates all doubt as to the presence of a definite cell wall in the living muscle and which is not produced in fixed and stained sections by artefactitious changes.[1])

Many workers, such as MALL, FLEMMING, SPALTEHOLZ, HANSEN, and others, have observed that the anlages of connective tissue fibrils are located within the genetic cell bodies. An adult fibril of such tissues is, however, either wholly extracellular or partly extracellular and partly intracellular. The names of ROLLETT, TOURNEAUX, MEVES and FERGUSON, in addition, may be mentioned in support of this latter statement.[2]) The same developmental sequence is observed in the instance of elastic tissue fibrillae. The work of HARRISON may also be cited, — „Die wichtigste Tatsache aus den vorgegangenen Beobachtungen ist, daß diese Skeletteile — die Hornfäden und Flossenstrahlen (of teleosts) — keinen intercellulären Ursprung haben, sondern das Ergebnis einer direkten Umbildung von gewissen Zellenteilen darstellen, welche von dem Mesenchym abzuleiten sind." The anlages of muscle fibrillae, as well, have an

[1]) In reviewing the literature it is not surprising to find that many of the published plates of striped muscle structure present what appears to be a muscle cell membrane. HEIDENHAIN's „Plasma und Zelle", contains such figures, as do also the following references to invertebrate muscle, LEYDIG's „Untersuchungen zur Anatomie und Histologie der Tiere", of 1883 and NUSSBAUM's „Anatomische Studien an californischen Cirripedien" of 1890. It is a remarkable fact, however, that none of these authors recognized the significance of the membrane nor the extracellular character of the muscle fibrillae.

[2]) SPALTEHOLZ has recently questioned this view, holding that the fibrillae of the adult tissues are still intracellular.

intracellular position as Heidenhain, Godlewski, Meves, Marceau, Bardeen, among others have demonstrated. My own studies of the adult, voluntary, striated, muscle fibres show that the voluntary muscle fibrillae have an extracellular position. The intermediate steps in histo-myo-genesis are as yet incomplete, still with the facts at hand derived from these two extremes, a seeming parallelism can be drawn between the histogenesis of the connective and elastic tissues on the one hand and that of the voluntary striped muscle fibrillae on the other. Accordingly it would appear to be justifiable to attribute to this form of muscle fibre a position among the connective tissue group of structures and not to regard it as a purely cellular structure. My conception of a voluntary striped muscle fibre is, therefore, not that of a gigantic, multinucleated cell, but rather of a composite contractile structure enveloped by a sheath, the sarcolemma, which encloses, in addition to the elements of contractility, muscle fibrillae, sarcoplasm, etc., muscle cells, which cells present all of the recognized features of general cell structure, cell membrane, cell protoplasm, consisting of spongioplasm and hyaloplasm, and a nucleus containing nucleoli.[1])

Zusammenfassung.

Die quergestreifte Muskelsubstanz ist nur anfangs eine intracelluläre Bildung und wird später extracellular verlagert, so daß Muskelzellen und fibrilläre Substanz mit dem zugehörigen Sarkoplasma getrennte Bestandteile werden. Die Histogenese des Bindegewebes und der Muskelfasern ist identisch.

Eine quergestreifte Muskelfaser ist somit, entgegen der bisherigen Auffassung, keine vielkernige Riesenzelle, sondern von viel komplizierterem Bau. Was bis jetzt Sarkolemm genannt wurde, umschließt Muskelzellen, Sarkoplasma und Muskelfibrillen. Die

[1]) There still remain to be solved many problems involving the voluntary muscle cells and fibres. A much greater number of lower vertebrate muscles should be considered. Not only cardiac muscle but unstriped fibres in the whole vertebrate series should be reviewed in the light of the facts which I have set forth. Other pertinent questions are the relation of the nerve fibrillae to these extra-cellular fibrillae and several aspects of the histo-physiological phenomena of contraction; the relation of the lymph spaces to the fibrillae; the extent of the muscle cells in a longitudinal direction; the position of the telophragmata relative to the muscle cells; etc., etc. Accordingly, my treatise can be considered to be but little more than a preliminary communication.

Zellen haben eine Membran; ihr Protoplasma besteht aus Spongioplasma und Hyaloplasma und umschließt einen Kern mit Nukleolen. Über die Natur des Sarkolemm wird in einer folgenden Abhandlung eingehender berichtet werden.

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Since the recent treatises of HEIDENHAIN and of KEIBEL and MALL contain good working bibliographies dealing with the questions involved in this investigation, I have considered it superfluous to append a long literature list. In addition to some works to which I have especially referred, I have added only those articles which have appeared since the time of writing of these two books.

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Explanation of figures of Plates VII—VIII.

Fig. I. This is an oblique section through an extrinsic muscle fibre of the eyeball of a white mouse fourteen days old. The preparation was stained especially deeply with Gages chloral hematoxylin. The cell membrane is everywhere distinctly seen with the attached spongioplasm fibrils. Magnification 1500 diameters.

Fig. II. This preparation is similar to the above. The core of protoplasm is, apparently, interrupted at a level slightly above the middle of the section. Magnification 1500 diameters.

Fig. III. This represents a muscle cell imbedded in a thigh muscle of an adult frog. Only the immediately adjacent muscle fibrillae are represented. The stain used was alcoholic hematoxylin, the fibrils of spongioplasm of the cell body, accordingly, lack the granular deposit of the chloral hematoxylin stain used on other preparations. The outline of the cell is most distinct. At the upper end of the sketch the internal surface of the obliquely-sectioned cell membrane with its attached fibrils of spongioplasm is to be observed. Magnification 1500 diameters.

Fig. IV. A preparation stained like the above and from the same muscle. The two groups of muscle fibrillae lying above and to the left are separated from the muscle cell by an artificial tear in the fibre. At the left side of the cell the torn edge of the cell wall can be seen to project into the clear space occasioned by the tear unaccompanied by sarcoplasm. Magnification 1500 diameters.

Fig. V. A portion of a longitudinal section of a muscle fibre of the thigh of an adult cat stained with alcoholic hematoxylin. A slight fold in the fibre occurring at the level of the nucleus explains the presence of the four transected muscle fibrillae. The muscle cell in this figure is closely applied to the internal surface of the sarcolemma. Its deeply stained protoplasm is very sharply marked of from the sarcoplasm of the muscle fibre by the cell wall. Magnification 1500 diameters.

Fig. VI. This is a portion of a thigh muscle of an adult cat sectioned obliquely and stained with alcoholic hematoxylin. The muscle cell rests against the investing sarcolemma. The distinct outline of the cell wall is not exaggerated in the sketch. The protoplasm of the cell body is very faintly stained and stands out in sharp contrast to the uniformly deep stain of the sarcoplasm. This sketch was made a little above the level of the center of the nucleus, consequently a space, occupied by cell protoplasm, intervenes between the latter and the cell wall. At the level of the nucleus similar cells show the nuclear membrane in contact with the cell membrane. Upon the sarcolemma-side of the cell the wall of the latter is fused with the sarcolemma. Magnification 1500 diameters.

Fig. VII. This is a transected extrinsic eye muscle fibre of a calf. The stain used was alcoholic hematoxylin. The sketch was made above the level of the center of the nucleus. The attachment of the cell wall to the sarcolemma is well shown. The intervening area of sarcoplasm between the cell wall and the muscle fibrillae has been carefully represented. Magnification 1000 diameters.

Fig. 1.

Fig. 6.

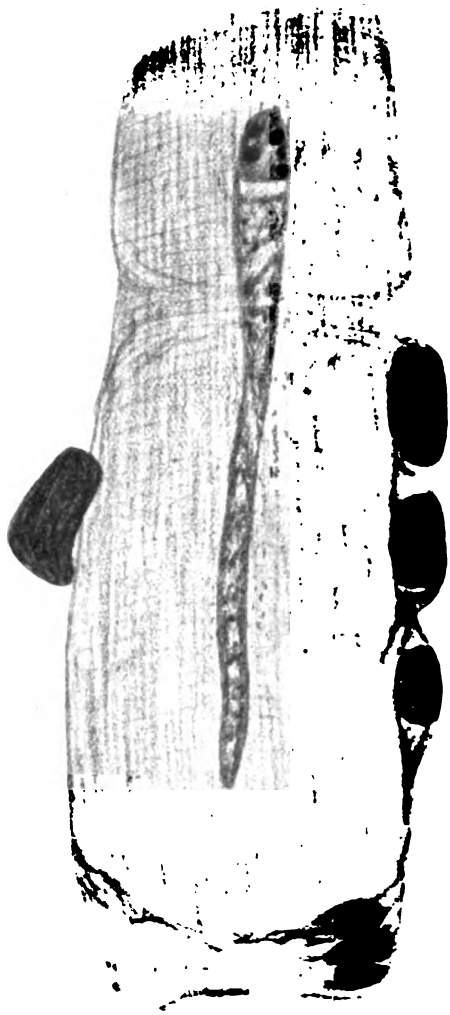

Fig. 2.

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Fig. 4.

Fig. 5.

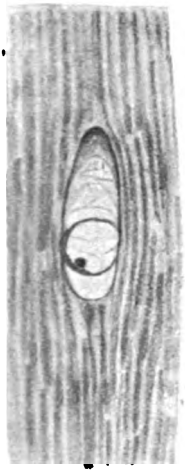

Fig. 3.

Fig. 8.

Fig. 9.

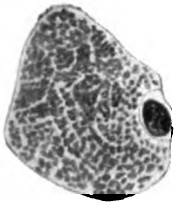

Fig. 7.

Fig. 10.

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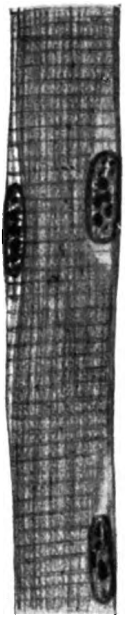

Fig. 11.

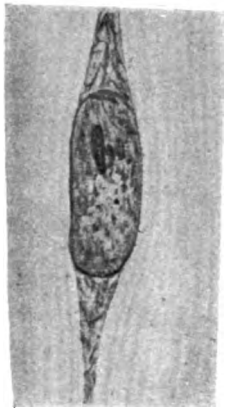

Fig. 13.

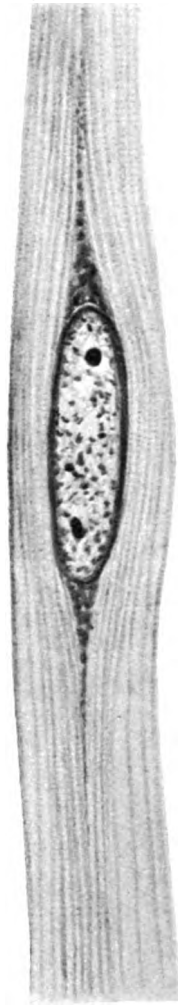

Fig. 12.

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Fig. VIII. This represents a transected fibre of the latissimus dorsi muscle of a three-weeks old chicken. The relations are the same as those described under fig. VII. Magnification 1000 diameters.

Fig. IX. This is a portion of a fibre of an extrinsic eye muscle of a calf cut longitudinally. Two muscle cells lying between the sarcolemma and the muscle fibrillae are represented. Notwithstanding the marked contrast between the structural features of the cell protoplasm and those of the sarcoplasm, because of the overlying and parallel running fibrillae it is impossible in this preparation to identify the cell membrane. I have projected the telophragma lines upon the same level with the cell protoplasm. They do not traverse this latter structure. Stain Gages chloral hematoxylin. Magnification 1000 diameters.

Fig. X. This is a portion of a fibre of the latissimus dorsi muscle of a three weeks-old chicken represented in longitudinal section. The telophragma lines can be seen to lie over the muscle cells. The stain used was alcoholic hematoxylin. The cell outlines, therefore, are clearly represented. Magnification 1000 diameters.

Fig. XI. This figure represents a longitudinal section of a fibre of an extrinsic eye muscle of a fourteen-day old white mouse, stained with chloral hematoxylin. Three muscle cells are seen. The general morphological appearances noted in figures IX and X are to be seen here as well. A muscle fibre traverses the face of the nucleus on the left. Magnification 1000 diameters.

Fig. XII. This is a muscle cell imbedded in a caudal muscle of a tadpole about 5,0 cm long. The immediately adjacent muscle fibrillae only are represented. At either end of the spindle-shaped cell body the protoplasm is drawn out to a pointed extremity. The determination of the exact extent of this protoplasm is very difficult, notwithstanding the marked differences between its structure and that of the adjacent sarcoplasm. The stain used was alcoholic hematoxylin and the magnification 1000 diameters.

Fig. XIII. This is a portion of a fresh thigh muscle of an adult frog showing a muscle cell imbedded among the muscle fibrillae. The fibre is represented in longitudinal section. The stain used was methylene blue. Everywhere the cell outline stands out in sharp contrast to the comparatively faintly stained muscle fibrillae. This specimen being a fresh preparation and not having been treated with any fixative reagents or other chemicals save the methylene blue stain alone represents practically a living cell and muscle fibre in which no shrinkage, contraction or other artefactitious change has yet appeared. The cell was located and the sketch outlined within ten minutes from the time of removal of the specimen of muscle from the living frog. Magnification about 1500 diameters.

The relation of the Sarcolemma to the Muscle Cells of voluntary vertebrate striped Muscle Fibres and its morphological nature.

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Cornell University Medical College New York City.

With plate IX.

(From the Biological Laboratory at Bonn.)

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In a recent communication[1]) I have set forth the conception as the result of studies upon various striated muscles that the voluntary striped muscle fibre of the adult vertebrate should be regarded not as a plasmodium containing many nuclei or a giant multinucleated cell, which view has been entertained up to the present day, but, rather, as a composite contractile structure consisting of muscle fibrillae and of sarcoplasm enclosed by sarcolemma and containing, in addition, many muscle cells. Each one of the numerous nuclei imbedded in the muscle fibre presents nucleoli, is immediately surrounded by differentiated cellular protoplasm consisting of strands of spongioplasm with interstices of clear hyaloplasm, and is completely and distinctly limited from the muscle fibrillae and the sarcoplasm of the muscle fibre by a cell membrane. In other words, every feature of cell structure is to be observed. The muscle fibrillae are, accordingly, extracellular, while the sarcolemma should not be regarded as a cell membrane in the sense that it envelopes a multinucleated mass of protoplasm. In my earlier contribution, however, I left unanswered many contingent questions, such as the structural position of the sarcolemma, its

[1]) Zeitschr. f. allg. Physiol., 1912.

exact relation to the enclosed muscle cells, the relation of the telophragma lines to such cells, the longitudinal extent of the latter, etc. The purpose of my present writing is to elucidate certain of these problems; in particular, however, the morphological relation of the sarcolemma to the muscle cell walls.

In accepting the claim that the muscle fibre contained complete cells and not merely isolated nuclei, questions as to the relation of the telophragmata to such cells, i. e., whether the cell protoplasm was traversed by the uninterrupted telophragmata, were immediately presented for solution. Granting that the telophragmata are septa which completely traverse the muscle fibre (HEIDENHAIN, CAJAL, MC CALLUM, RENAUT, MARCEAU, HOLMGREN, etc.) then at the level of the muscle cells either the protoplasm of the latter is traversed or the telophragmata are interrupted. In the latter contingency especially in reference to the peripheral muscle cells the views entertained by M. HEIDENHAIN, MARCEAU, RENAUT, HOLMGREN, ZIMMERMANN, that the telophragmata are directly attached to the sarcolemma must suffer some exceptions or the sarcolemma must be looked upon as surrounding each muscle cell in addition to investing the fibre as a whole. Nothing, so far as I know, has been stated definitely by the various investigators considering these problems regarding the traversal of the differentiated protoplasm investing the muscle nuclei. Indeed, the true nature of this protoplasm has been, as well, overlooked. At the level of the nuclei, however, i seems to be generally admitted, and is well represented in many of the figures of the workers whom I have cited, that the telophragmata do not traverse the muscle nuclei.

This present contribution is based upon studies of various voluntary muscles of Axolotl, chicken, white mouse, and cat. The technic and staining methods employed are the same as I described in my preliminary contribution. The secret upon which the derivation of good results depends is none other than overstaining with long prolonged extraction. In the present study I have confined my attention to those muscle cells which occupy a peripheral position in the muscle fibre lying in contact with the sarcolemma. The results present additional evidence bespeaking the new conception of the voluntary striped muscle fibre, which I have outlined above, and verify with increased emphasis the fact that the muscle fibrillae are extracellular, or intercellular, structures in the adult.

In order to present clearly and consecutively the fundamental facts ascertained by my studies, I have selected a series of white

mouse preparations which are represented in figs. 1 to 4, inclusive. These are fibres from a thigh muscle of a three-fourths grown white mouse and are sketched at a magnification of 1000 diameters. The features noted in this series are to be taken not as limited to the muscles of this form of vertebrate, but, rather, as typical of the general condition found and as well presented in all of the other vertebrate muscles which I have studied.

In fig. 1 a portion of such a muscle fibre cut in its long axis is represented. The free edge of the fibre presents a single muscle cell with its relatively large nucleus. The muscle fibrillae are seen to be deviated slightly out of their course in their passage by the nucleus. Some of these fibrillae lie upon an optical plane lower than the level of the nucleus. One fibril is represented as traversing the face of the nucleus. In brief this peripheral muscle cell occupied that aspect of the muscle fibre turned at right angles to the observer. The presence of the bulgings of the sarcolemma between the attachment of the telophragmata noted upon the right aspect of the fibre may be accepted as evidence of the fact that the fibre was fixed in a condition of contraction. By reason of these bulgings the relation of the sarcolemma to the cell can be all the more readily observed. It is seen to pass across the face of the nucleus. Careful focusing demonstrated that the same relation obtained on the under aspect of the cell.

The specimen was very deeply stained with chloral hematoxylin, so deeply, in fact, that those aspects of the sarcolemma but slightly inclined towards or away from the eye could be readily observed as distinct laminae. Therefore it was possible to see at the level of the serrated edge of the sarcolemma traversing the face of the nucleus, two surfaces of sarcolemma, one upon the uppermost optical level of the fibre and the other between this and the muscle cell. Indeed, the continuity of these two surfaces could be readily observed at the serrated edge. Furthermore, the telophragmata could be followed in unbroken continuity from the uppermost aspect to the lower around the serrated edge. Between these two aspects lay the muscle fibril which I have represented and to which I have previously referred. The sarcolemma was, in fact, indented by the muscle cell and lay between the latter and the muscle fibrillae at the level of the center of the muscle cells. It did not enclose the muscle cell. At the level of the center of the muscle nucleus, because of its proximity to the nuclear membrane, its identity was obscured.

These same relations of sarcolemma, of telophragmata, and of muscle cell could be seen upon the under aspect of the fibre. As I have mentioned above, the general appearance presented was that of an indentation of the fibre by the muscle cell.

The question naturally arising as to whether the cell under consideration was a connective tissue cell overlying or underlying the muscle edge is negatived by the relations of sarcolemma which I have already outlined. Further support is contributed by a study of the relationship of the protoplasm of the extremities of the cell, (c).

In such positions the serrated border of the sarcolemma occupies the extreme edge of the muscle fibre. At such levels, too, the cell protoplasm lies among the muscle fibrillae as I have represented, in other words, is encompassed by the sarcolemma and the telophragmata.

Fig. 2 represents a portion of a muscle fibre containing a muscle cell similar to that in fig. 1 but rotated through an arc of 90° so that the cell occupies the uppermost aspect of the fibre and consequently faces the observer. Many of the conditions unexplained in the foregoing figure are readily understood by a study of this sketch. There is no question as to its being a muscle cell and not a connective tissue cell. It is buried in the muscle fibre. The telophragmata are observed to mark off the bulgings of the sarcolemma upon either border of the muscle fibre. They do not, however, pass across the surface of the nucleus. Herein lie the characteristic and significant features of the specimen.

Overlying the muscle cell the serrated edge of the sarcolemma is observed to enclose a spindle-shaped space. The telophragmata proceed only up to this serrated edge. In this deeply-stained hematoxylin preparation the sarcolemma can be seen to be folded back upon itself in order to pass around the cell body. Hence the presence of the serrated interval, hence, also, the absence of the telophragmata upon the face of the cell. The sarcolemma does not traverse this uppermost aspect of the cell. It is reflected, rather, between the muscle fibrillae and the cell. In other words, it is invaginated into the muscle fibre by the cell.

I assured myself that the spindle-shaped space was not owing to the passage of the section knife through the uppermost aspect of the fibre by a most careful study of similar chloral-hematoxylin preparations which were especially deeply stained. In these the sarcolemma as a distinctly stained and homogenous membrane could

be seen to be reflected upon itself at the serrated edge. There was no indication whatever of an artefactitious tear or section cut in the fibre.

In figs. 3 et 4 similar muscle fibres containing peripheral muscle cells are represented in transection. An entire muscle fibre and a portion of another are seen in figure 3. Each contains a muscle cell. That at A was encountered by the section knife at a level corresponding to A in fig. 2, hence the nuclear membrane, cell membrane, and immediately investing sarcolemma appear to be one. The nucleus seems to be imbedded in and in direct contact with the sarcoplasm of the fibre. Such appearances I have commented upon more fully in my other paper.

The cell indicated at C was transected at a level corresponding to level C in fig. 2. Nothing of the nucleus is to be seen; strands of spongioplasm, however, traverse the protoplasmic substance of the cell which is invested by cell wall plus sarcolemma.

This fact must be especially noted. According to our interpretation of the appearances presented by the cell in fig. 2 the nucleus and cell wall lie in direct contact with the perimysium of the muscle fibre, i. e., the sarcolemma does not lie upon the peripheral aspect of the cell, but, on the contrary, is reflected between it and the muscle fibrillae. The protoplasmic extremity of the cell at C, however, does not lie upon the periphery of the fibre, but passes deeper and deeper into the substance of the muscle fibre lying among the fibrillae. A narrow interval of sarcoplasm, hence, intervenes between the fused perimysium and sarcolemma encompassing the muscle fibre as a whole and the fused cell wall and sarcolemma immediately investing the cell protoplasm. Just how this sarcolemma comes to be invaginated into the muscle fibre among the fibrillae is rendered clear by a reference to diagram 5. Of these features I will speak more at length later.

The nucleus in fig. 4 has been sectioned at the level B (fig. 2), i. e., through one of its poles. At this level the nuclear membrane is separated from the cell wall and sarcolemma by an interval of cell protoplasm. It does not lie in contact with the investing membrane of the muscle fibre. The place of infolding of the sarcolemma to invest the nucleus is well represented at X. In the rest of its extent about the muscle fibre, its identity as a distinct membrane is lost because of its fusion with the investing perimysium. The place of infolding is the serrated edge observed in longitudinal sections (fig. 2) and encloses the cigar-shaped space

referred to under the latter figure. Several muscle fibrillae occupy the angles of reflection. These were observed in figs. 1 and 2 to traverse the face of the nuclei. By reason of these infoldings the muscle cells really lie outside of the muscle fibre. In other words, the muscle fibrillae are intercellular. The muscle cells are merely received into a depression in the muscle fibre, lined with sarcolemma while the extremities of the cells penetrate among the fibrillae in tube-like prolongations of this same sarcolemma (c, fig. 3). The fact should be particularly noted in this specimen, since it is well demonstrated, that the sarcolemma is wanting between the muscle cell and the investing perimysium of the fibre opposite the level of the nucleus.

This interpretation of the relation of the sarcolemma to peripheral muscle cells renders reasonably clear the significance of the relation of the telophragmata to such cells. It is a significant fact that none of the figures published represent these lines as transecting the nuclei. In general they are represented as terminating abruptly in the immediate vicinity of the nuclei. Undoubtedly, because of too faint staining, both the infoldings of the sarcolemma at the edges of the nuclei and the presence of the serrated edge enclosing the cigar-shaped interval overlying the face of the nuclei have been overlooked.

The sketch 5 is a semi-diagrammatic representation of a muscle fibre cut longitudinally exactly through the middle of a peripheral muscle cell. The sketch is intended to demonstrate the relation of the sarcolemma to the cell wall. For the purpose of making this relation clearer the cell wall, C, is drawn with a narrow interval separating it from the sarcolemma, A—B. For the sake, also, of clearness the cell protoplasm with its fibrillae of spongioplasm is omitted. Consequently, the nucleus appears to lie in a clear space.

Tracing the sarcolemma, A, of the periphery of the fibre towards the nucleus, we note that at F it is reflected and passes then in contact with the cell wall, C, back to A. The angle F is the serrated edge outlining the cigar-shaped interval mentioned above. It contains, as shown, two muscle fibrillae cut obliquely which diverged out of the exact long axis of the fibre in order to proceed uninterruptedly around this interval. Between the angles of reflection of the sarcolemma, the cell wall lies in direct contact with the perimysium, D. That this last named structure contains nuclei is shown by the cell represented at E upon the left border of the fibre. Many of such spindle-shaped cells are to be found throughout

the entire muscle series. But little doubt exists, then, that the perimysium is a connective tissue structure. As yet, however, I have failed to find a single nucleus in the true sarcolemma.

A portion of two muscle fibres cut longitudinally is represented in fig. 6. These are from a thigh muscle of an adult cat. By reason of a tear in the specimen the two fibres are separated from each other by an interval which is bridged, nevertheless, by several connective tissue strands. The fibre on the left contains two muscle cells united by cellular protoplasm which tunnels the peripheral portion of the muscle fibre. The relations of the crenated sarcolemma edge to the nuclei and of the perimysium to the upper muscle cell are the same as those noted in connection with the foregoing figures and, hence, require no further amplification. The lower of the two cells, however, presents several elucidating features.

The crenated border of the sarcolemma passes across the face of this nucleus. Because of the separation of the two fibres, however, the fibrillae constituting the perimysium are pulled away from the cell body and from the lower portion of the muscle fibre. One of these fibrillae is seen to be derived from a connective-tissue cell. Hence, the lower portion of the muscle fibre is naked so far as its perimysium investment is concerned. The sarcolemma alone clothes this portion of the fibre, and in it, incidentally, no cells are to be found. The removal of the perimysium has left the cell wall bare. The delicate nature of this structure, therefore, can be seen. In the extreme lower end of the sketch the cell protoplasm is observed to pass into the interior of the muscle fibre. Careful focusing and a study of the relation of the telophragmata to this protoplasmic mass proved that this cell extremity lay imbedded among the fibrillae of the muscle fibre. This specimen furnishes the most conclusive evidence upon the identity of the sarcolemma as a homogenous membrane, of the perimysium as a connective tissue structure, and of the presence of a distinct cell wall of the muscle cells.

The following four sketches are presented for the purpose of demonstrating the intimacy of contact of the sarcolemma with the perimysium. The first two figures are precisely similar to such transections which are ordinarily represented in text-books. The sketch (fig. 7) was taken from a section of thigh muscle of a white mouse three-fourths grown and demonstrates portions of four muscle fibres transected. The nucleus at A is that of a peripheral muscle cell that at B, a perimysium cell. The latter possesses a spindle-

shaped body from whose extremities the perimysium passes off to encircle the muscle fibre.

Fig. 8 is a portion of a transected thigh muscle of an adult cat. The cell at A is a connective tissue cell and gives off several processes. The cell at B is similar to the cell indicated at B in the foregoing figure, a spindle-shaped perimysium cell whose processes are incorporated in that structure. In the instance of both of these perimysium cells the observation can very readily be made that, apparently, at least, the perimysium splits at the cell to enclose it, yet the internal of the two layers formed by this splitting process is so thin that, opposite the middle of the cell nucleus, its presence cannot be determined. Indeed, between the nucleus and the sarcoplasm no formed structures can be discerned, yet our studies of longitudinal sections, as we have seen above, show that the exceedingly thin sarcolemma does lie between the fibrillar, cell-containing perimysium and the sarcoplasm. Its thinness and its intimacy of contact with the perimysium are such that, only in those places where it leaves the latter to be reflected around the peripheral muscle cells, can its identity be recognized.

That this intimacy of contact with the perimysium is real and not apparent, the last two sketches demonstrate. The muscle fibre in fig. 9 is a transected trunk muscle of Axolotl and that in fig. 10 a similar section of the latissimus dorsi of a three-weeks old chicken. In both specimens, tears had been caused. As a result the perimysium was pulled away from the sarcoplasm. It is to be noted that the sarcolemma adheres to the former. The evidence for this fact must be given negatively, since is not demonstrable upon the border of the sarcoplasm imbedding the muscle fibrillae. This is seen to be rather ragged in outline such as would not be expected were the sarcolemma in situ. The appearances in fig. 10 seem to negative this latter statement, however, since the free edge of the sarcoplasm appears to possess a sharply defined thickened border, — a true sarcolemma. It must be noted in the sketch, however, that a small lappet upon the left aspect of the sarcoplasm indicates that a tear through the latter had been occasioned in the specimen. As a result a group of thirteen or fourteen fibrillae with their imbedding sarcoplasm was pulled away from the main fibril group. The significant fact demonstrated by the specimen is that in the angle of this tear the same apparently sharp edge of the sarcoplasm is observed. In other words, the edges of the torn sarcoplasm present the same distinct border as is observable elsewhere

upon the surface of the sarcoplasm. Therefore, it is very probable that this seeming border is not sarcolemma but an artefactitious product, a kind of superficial increase in density of the sarcoplasm.

The remarks which I have made above and the conclusions which I have drawn, apply particularly to peripheral muscle cells. I feel sure by induction, however, that the same relation of sarcolemma to cell wall and of cell wall to cell protoplasm obtains in the instance of central-lying muscle cells. In fact, I have observed many such cells encompassed by sarcolemma. Until a new negative staining method is devised by which the cell protoplasm is stained and the muscle fibrillae unstained, however, I cannot commit myself upon the derivation of the sarcolemma immediately investing the cell wall, i. e., whether it represents a portion isolated from that enveloping the fibre or is derived from the same in a tube-like process tunneling the length of the muscle fibre. So far as I have gone with my research studies the latter appears to be the case, yet the evidence is not so coherent as to be presented in an absolutely convincing whole, by reason, as I have mentioned above, of a staining difficulty. In brief, the fundamental problem involved is the determination of the exact extent of the protoplasmic masses belonging to the imbedded cells. The difficulties present in the solution of this problem still await solution.

I have not yet observed a single centrally-located muscle cell, however, whose protoplasm was traversed by the telophragmata of the muscle fibre. I am in a position further to corroborate fully the views entertained by HEIDENHAIN, SCHIEFFERDECKER, MC CALLUM, ENDERLEIN, HOLMGREN, ZIMMERMANN, among others, that in the adult striped muscle the telophragmata are directly attached to the sarcolemma. The only interruptions occurring in this attachment are apparent rather than real and are to be noted in connection with the protoplasmic masses of the central and of the peripheral muscle cells. In such instances the telophragmata are directly attached to the sarcolemma immediately investing these muscle cells. They do not traverse either the protoplasm or the nucleus of such cells. Such observations as those of ENDERLEIN on Gastrus equi and on Hypoderma Diana, where the telophragmata traversed the protoplasmic cellular masses outlying the muscle fibrillae but enclosed within the sarcolemma, I have failed to verify in the adult muscles of the higher vertebrates which I have studied

Without entering too extensively into the history of the investigations upon the nature and relations of the sarcolemma, the following

brief statements of the more pertinent facts should be made. As early as 1840 BOWMAN named the structure, which we have generally considered as sarcolemma, and described it as a structureless membrane serving as an envelope for the muscle fibres. REICHERT subsequently questioned this view of its morphological character, however, maintaining that it was a connective tissue structure and contained nuclei. These two papers marked the beginning of a half-century dispute which has lasted down to the present day. The credit belongs to LEYDIG, however, for first advancing the view of the sarcolemma of the vertebrate muscle as a cuticular structure. CALBERLA also held the same opinion. HENLE, on the contrary, found nuclei in it. CAJAL, HOCHE, HEIDENHAIN, MARCEAU, RENAUT, ZIMMERMANN, and HOLMGREN concluded, in spite of the fact that they could discern fibrils and cells in the sarcolemma, that in cardiac muscle fibres the latter should be regarded as a cuticular thickening of the sarcoplasm. One of the most recent contributors upon the subject is SCHIEFFERDECKER. This investigator utilized the trunk musculatur of Petromyzon for his purposes. In the "central" fibres of such muscles he found the examples, long desired by investigators, where sarcolemma, devoid of perimysium, alone enveloped the fibres. In the study of such specimens he came to the conclusion that the sarcolemma of the voluntary striated muscle was a homogenous cuticular thickening and possessed neither cells nor fibrils. I, too, have studied such specimens and, as well, have arrived at the same conclusions. I differ in opinion from the latter and from MAURER, among others, however, in regarding this thickened cuticula not as a cell membrane. It is apparently a derivative of the sarcoplasm, by a process of condensation and in this respect alone resembles a cell membrane. It does not, however, encompass a multinucleated mass of protoplasm, since the sarcoplasm imbedding the muscle fibrillae, as I have demonstrated above does not contain nuclei.

My own studies have demonstrated in addition, that in the muscles which I have utilized and under the conditions which I have enumerated the sarcolemma of the adult, striped, voluntary muscle can be observed as a homogenous, fibre-less, and cell-less membrane.

The cause of the long controversy upon the subject is referable to the fact that the structure seen in ordinary transections of voluntary striped muscle fibres, as figures 7 and 8 show, is not sarco-

lemma but sarcolemma plus perimysium. The cells and fibres detected in it belong not to the sarcolemma but to the perimysium.

The conclusions to which I have arrived, then, as the result of my studies upon these adult voluntary striated vertebrate muscles are:

1. The sarcolemma is a thin, structure-less, homogeneous membrane completely investing the muscle fibre.

2. It contains no cells and no fibrillae.

3. It is infolded from the perimysium to invest the peripheral muscle cells.

4. At such places it separates the muscle cells from the sarcoplasm of the muscle fibre.

5. At such places, likewise, the muscle cell wall opposite the level of the nucleus lies in direct contact with the perimysium.

6. Upon its internal surface the sarcolemma is always in direct contact with the sarcoplasm.

7. The telophragmata are always directly attached to its internal surface.

8. Its external surface is fused with the perimysium.

9. The sarcolemma and the perimysium together constitute the investing envelope of the muscle fibre.

10. Only at the position of the peripheral muscle cells are these two structures separated from each other.

11. The perimysium is a connective tissue structure containing both spindle-shaped cells and fibres.

12. The sarcolemma is so thin and so closely applied to the perimysium as to escape observation in transections excepting at those levels where it is infolded to invest the peripheral muscle cells.

13. It can be seen to best advantage as a separate layer in chloral-hematoxylin preparations and at those levels in longitudinal sections where it is infolded to invest the peripheral muscle cells.

14. The telophragmata do not traverse the protoplasm of the muscle cells nor their nuclei.

All of these results are merely confirmatory of my earlier observations, i. e., — that the voluntary, striped muscle fibre of the adult vertebrate is not a giant, multi-nucleated cell, but, on the contrary, a composite contractile structure consisting of muscle fibrillae and of sarcoplasm enveloped by sarcolemma, and, in addition, numerous muscle cells. In other words, the muscle fibrillae are extracellular structures. The muscle nuclei are not directly imbedded in the sarcoplasm of the fibre but are separated from it

by a layer of sarcolemma and by cell protoplasm which possesses a distinct cell wall. The terms muscle fibre and muscle cell as applied to this form of striped muscle are not synonymous. Furthermore, the muscle fibrillae are comparable to the fibrillae of adult connective tissues to the extent, at least, that both are intercellular in position.

Zusammenfassung.

Das Sarkolemm ist eine dünne, strukturlose, homogene Hülle der Muskelfaser ohne Zellen und Fibrillen. Seine äußere Oberfläche schmiegt sich dem Perimysium an und nur da, wo die Muskelzellen gelegen sind, weicht es von dem Perimysium zurück, so daß die Muskelzellen zwischen Perimysium und Sarkolemma liegen und das Sarkolemm nur das Sarkoplasm und die Muskelfibrillen umschließt.

Die Telophragmata sind mit der inneren Oberfläche des Sarkolemms verwachsen und dringen nicht in das Protoplasma der peripheren Muskelzellen ein.

Das Perimysium ist bindegewebiger Natur; es enthält Fasern und spindelförmige Zellen.

Perimysium und Sarkolemm zusammen umgeben die Muskelfaser und sind nur dort voneinander getrennt, wo die peripheren Muskelzellen gefunden werden. Das Sarkolemm ist so dünn, daß es an Schnitten der Beobachtung sich entzieht, außer an den Stellen, wo es in der Gegend der peripheren Muskelzellen von dem Perimysium sich zurückklappt und eine Mulde für diese Zellen bildet. Am besten kann das Sarkolemm an Chloral-Hämatoxylinpräparaten sichtbar gemacht werden.

Somit sind die Muskelfibrillen extrazelluläre Bildungen. Die Muskelzellen sind vom Sarkoplasma und den Muskelfibrillen durch das Sarkolemm getrennt und gemeinschaftlich mit diesen beiden Bestandteilen der Muskelfaser vom Perimysium umgeben. Die Muskelzellen haben eine deutliche Zellmembran. Die Muskelfibrillen sind den ausgebildeten Bindegewebsfibrillen vergleichbar, da beide extrazellulär gelegen sind.

Muskelfaser und Muskelzelle sind nicht synonym, soweit es sich um die hier untersuchten Arten quergestreifter Muskeln handelt.

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Explanation of figures of Plate IX.

Fig. 1. A longitudinal section of a portion of a thigh muscle of a three-fourths grown white mouse. A peripheral muscle cell with its prominent nucleus is located upon one side of the fibre. A indicates the level of the center of the nucleus; B the extremity of the same where the nuclear membrane lies at some distance from the cell wall; C the protoplasmic extremity of the cell prolonged into the muscle fibre among the fibrillae. Magnification 1000 diameters.

Fig. 2. A surface view of a peripheral muscle cell of a muscle fibre the same as that in fig. 1. The serrated edges of the reflected sarcolemma traverse the face of the nucleus leaving a space between in which the muscle cell wall lies in direct contact with the perimysium. The letters A, B, and C correspond to those of fig. 1. Magnification 1000 diameters.

Fig. 3. A transection of two muscle fibres derived from the same source as that of fig. 1. At A a peripheral muscle cell is represented cut at the level indicated by the same letter in figs. 1 and 2. The nucleus lies in direct contact with the cell wall. At C another peripheral muscle is seen. This was encountered at the level C of figs. 1 and 2, i. e., only the protoplasm of the cell prolongation is seen. A layer of sarcolemma invests the cell wall. A narrow interval of sarcoplasm intervenes between this and the investing sarcolemma and perimysium of the muscle fibre, P. Magnification 1000 diameters.

Fig. 4. Similar to fig. 3. At B a muscle cell cut transversely at the level B of figs. 1 and 2 is seen. A narrow interval of protoplasm intervenes between the nucleus and the cell wall. At x the sarcolemma is reflected from its position in contact with the perimysium to invest the muscle cell, thereby separating it from the sarcoplasm and fibrillae of the muscle fibre. Magnification 1000 diameters.

Fig. 5. A semidiagrammatic sketch of a surface view of a portion, of an adult voluntary striped muscle fibre cut through the median longitudinal axis of a peripheral muscle cell. The cell wall, C, sarcolemma A—B, and perimysium, D, are separated from each other by a narrow interval. The protoplasm of the cell is omitted. F, the angle of infolding of the sarcolemma by which is produced the serrated edge noted in fig. 2 as traversing the face of the nucleus. Between the two angles of reflection (upper and lower in the figure) the cell wall lies in immediate contact with the perimysium. At E a spindle-shaped cell of the perimysium is shown. Magnification about 1500 diameters.

Fig. 6. A portion of two adjacent thigh muscle fibres of an adult cat cut in their long axis. Between the two fibres the interval, occasioned by handling of the tissue, is traversed by several strands of perimysium fibrils. At the lower end of this interval a connective tissue cell receives one of these fibrils. Two peripheral muscle cells occupy the right border of the muscle fibre and are connected with each other by a strand of protoplasm. The serrated edge of the sarcolemma traversing the face of the nuclei is well shown. At D the perimysium is torn away from the muscle fibre leaving the cell membrane at C bare. Magnification 1000 diameters.

Fig. 7. In this sketch a portion of four muscle fibres of a thigh muscle of a three-fourths grown white mouse is shown. The fibres are transected and demonstrate two nuclei, the one on the left being that of a peripheral muscle cell cut at the level A of figs. 1 and 2 and for this reason not demonstrating either the cell wall or the sarcolemma intervening between it and the sarcoplasm, while that on the right is a spindle-shaped perimysium cell. Between this latter cell and the enclosed sarcoplasm of the fibre the sarcolemma cannot be made out by reason of its thinness and the closeness of application to the perimysium. Magnification 1500 diameters.

Fig. 8. This represents a portion of a transected muscle of the thigh of an adult cat. A connective tissue cell with several fibres is adjacent to the perimysium which presents a spindle-shaped cell. Between the latter and the sarcoplasm the sarcolemma cannot be seen as a distinct layer.

Fig. 9. This figure is presented to show that, in a transected voluntary muscle of Axolotl where the investing envelope has been torn away by mechanical means from the fibrillae, the adhesion between the sarcolemma and the perimysium is greater than that between the former and the sarcoplasm. A bipolar perimysium cell is seen in the torn envelope. The sarcolemma, exceedingly thin and though not demonstrable upon the internal surface of the perimysium because of its intimate incorporation with the latter, is very manifestly not present upon the ragged, and irregular edge of the sarcoplasm. Magnification 1000 diameters.

Fig. 10. A transected fibre of the latissimus dorsi muscle of a three-weeks old chicken. The features demonstrated are similar to those of fig. 9. Magnification 1500 diameters.

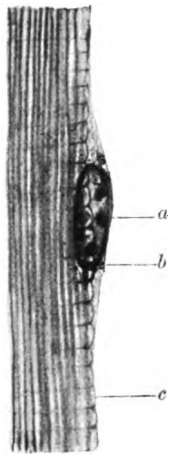

Fig. 1.

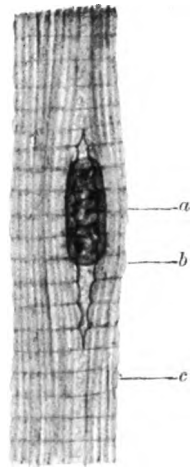

Fig. 2.

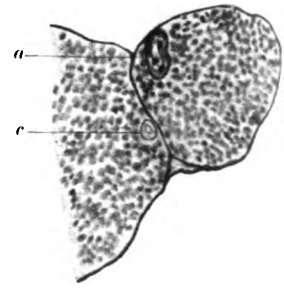

Fig. 3.

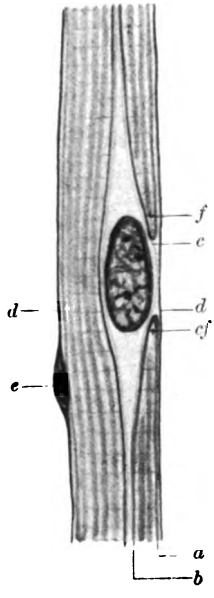

Fig. 5.

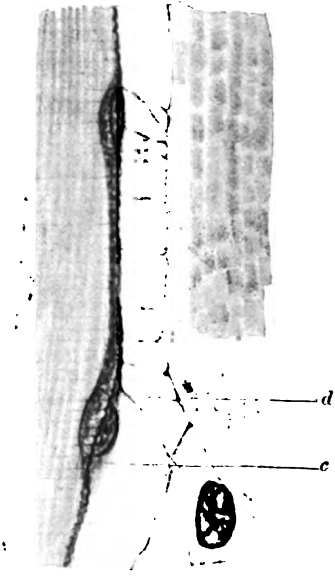

Fig. 6.

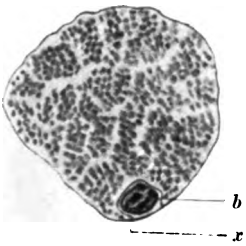

Fig. 4.

Fig. 7.

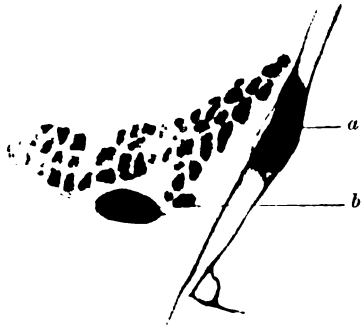

Fig. 8.

Fig. 9.

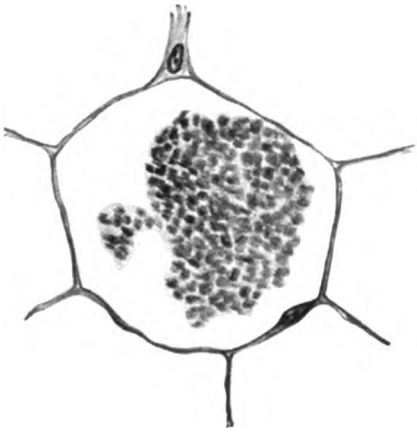

Fig. 10.

Fischer in Jena.

(From the Biological Laboratory at Bonn.)

The Relation of Muscle Fibrillae to Tendon Fibrillae in voluntary striped Muscles of Vertebrates.

By

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With plate VII.

The long-contended question over the structural relationship of tendon fibrillae to muscle fibrillae has been brought to our attention by a recent contribution of O. SCHULTZE. This investigator carried out his studies on various muscles of *Hippocampus, Amphioxus*, several amphibia, and man, and arrived at the conclusion that these two structural features, muscle fibril and tendon fibril, were directly continuous with each other, perforating the sarcolemma. Being at the time engaged in a study of certain morphological features of striped-muscle structure, I reviewed my own preparations demonstrating these features with the express purpose of advancing our knowledge in this subject over a larger number of higher vertebrates than this author had used.

The preparations comprised sections of various muscles, such as intercostales, latissimus dorsi, rectus abdominalis, gastrocnemius, erector spinae, extrinsic muscles of the eyeball and various muscles of the thigh (together with caudal muscles) of such vertebrates as the tadpole, frog, calf, cat, white mouse, chicken, gray mouse. In addition I utilized specimens of living muscle of the frog and of the tadpole as controls to the fixed and stained preparations. The paraffin method of imbedding was employed in conjunction with SCHULTZE's excellent collodion and chloroform method of infiltration. The sections varied in thickness from 2 μ to 5 μ.

The stains used were picric acid, methylene blue, fuchsin S, and eosin, together with combinations of these and various alcoholic

and aqueous solutions of hematoxylin among the latter SCHULTZE's and also GAGE's. Several of the more significant of the preparations were stained, decolorized, and then restained by another method in order not only to serve as controls to the simple stained sections but to demonstrate, in addition, the reaction of the several structures under consideration to the various methods previously employed. By means of a method of blunt dissection of the fixed and stained preparations upon the slide, several muscle fibres with their tendons were isolated from adjacent structures, by which was rendered possible a more careful and detailed study of their structural relationship.

In fig. 1 I have represented such an isolated muscle fibre with its attached tendon of an extrinsic eye muscle of a three-weeks old chicken. The instance is typical of the usual fibre-termination observed in these muscles. It can be seen that the sarcolemma at its extremity is drawn out to three distinct pointed processes which are not in any way continuous either with the sheath of the tendon or with the fibrillar components of the tendon. Furthermore, the pointed extremities of the tendon fibrillae are observed to be inserted into the recesses between the sarcolemma processes. This feature is worthy of special attention because owing to it an appearance is produced, when such an arrangement is rotated and studied in a vertical optical plane, of tendon fibrillae lying inside of the sarcolemma sheath of the muscle fibre. The individual muscle fibrillae, upon approaching the sarcolemma, lose their several features of cross-striation but can be traced, however, as slender, faintly-stained thread-like structures up to the internal surface of the sarcolemma upon which they terminate. The sarcolemma is in general much thinner than the cross-diameter of an average muscle fibril. Hence from the morphological arrangement of the parts thus effected, the muscle fibre appears to be dovetailed into the tendon with this exceedingly thin membrane as the only structure separating the muscle fibrillae from the tendon fibrillae.

Fig. 2 is another fibre from the same muscle, and demonstrates the same general features. Two muscle nuclei imbedded in granular protoplasm separate two groups of muscle fibrillae from each other. Each group, however, terminates in several pointed sarcolemma processes which are dovetailed with the fibrils of the corresponding tendon-fibril groups. Here again the muscle fibrillae, losing gradually their features of cross-striation, are, nevertheless, readily trace-

able up to the internal surface of the sarcolemma. There is no evidence, however, either upon morphological or upon staining-reaction grounds for the assumption that the fibrillae of muscle and tendon penetrate the sarcolemma. Neither can it be demonstrated that the sarcolemma is prolonged over the tendon or among its constituent fibril bundles. It terminates bluntly, rather, in a number of cone-shaped processes.

Fig. 3 represents the termination of an extrinsic muscle of the eyeball of a twenty-two-day-old white mouse. The observations made above in connection with the chicken muscles apply equally well to the muscles of this group in the mouse. Again, there is observed, the dovetailing of the sarcolemma processes with the tendon fibrillae, the termination of the muscle fibrillae at the sarcolemma, and the separation by the latter of the tendon fibrillae from the former.

I particularly desire to refer again to the insertion of the tendon fibrillae into the intervals between the cone-shaped prolongations of the sarcolemma through which arrangement certain appearances are produced which are exceedingly liable to be misinterpreted. This thin membrane is the only structure separating the muscle fibrillae from the tendon fibrillae. The latter pursue a course parallel to that of the former but terminate bluntly upon the external surface of the sarcolemma, whereas the muscle fibrillae run for a comparatively short distance upon the internal surface of the sarcolemma and finally lose their identity by fusing with it. Such conclusions can only be drawn from a study of those fibrillae which lie upon the same horizontal optical plane, i. e., upon that aspect of the sarcolemma which faces at right angle to the observer. When the muscle fibres and tendon fibres are cut in exactly their long axis, one must bear in mind that the uppermost and undermost aspects of the cone-shaped sarcolemma end are obliquely inclined to the vertical optical axis of the observer. This fact adds to the difficulty of interpreting the relation of the fibrillae more so than would be the case if the sarcolemma surface lay in a horizontal optical plane. Hence the solution of the question is much dependent upon the manipulation of the fine adjustment screw. Bearing in mind the fact that the sarcolemma is so thin as to be almost perfectly transparent, when studied upon these aspects of the fibres, the difficulty in determining the exact relationship of the muscle fibrillae to the overlying or underlying and parallel-running tendon

fibrillae is well nigh impossible with our present optical instruments. When viewed in such vertical planes it appears as if those tendon fibrillae terminating in the sarcolemma indentations were really within the muscle fibre. Undoubtedly a neglect to take into consideration these facts has led to several erroneous conclusions such as are represented in various published figures. Accordingly, I have preferred to base my conclusions upon a careful study of those fibrillae which occupy the same horizontal optical plane. Under such circumstances the sarcolemma presents an unbroken contour. No evidence can be found bespeaking a continuity of tendon fibril with muscle fibril among these various extrinsic eye muscles of the white mouse, gray mouse, chicken, or calf.

One of the best bits of evidence upon this question is furnished by muscles of the bipenniform type. I have represented in fig. 4 a portion of a single muscle fibre of this type of an adult white mouse with its attached central tendon. As is readily seen the tendon fibrillae (*A*) lie at an angle of about 125° with the muscle fibrillae (*C*) The sarcolemma investing the fibre is considerably thickened at that end applied to the tendon. The numerous muscle fibrillae are represented half-schematically but their relation to this sarcolemma end is faithfully reproduced. Between the sarcolemma and the tendon there are to be seen several layers of connective tissue fibres and cells (*B*). I was able by means of various methods of hematoxylin staining to stain at once these three structures, central tendon, intervening connective tissue, and muscle fibrillae three different colors upon the same slide. The tendon fibrillae were yellowish-white, the connective tissue (peritendinum) reddish-brown, and the muscle fibrillae deep brown. There was absolutely no structural continuity to be seen between either the tendon fibrillae and the connective tissue fibrillae or between the latter and the muscle fibrillae. At no place could it be demonstrated that the muscle fibrillae traversed the connective tissue sheath in order to reach the central tendon, nor did they perforate the sarcolemma and turn at an angle to join the connective tissue fibrillae.

In muscles belonging to this type of structure the relation of the muscle fibres to the tendon is precisely similar to that of those muscles which are attached to bones, to the bone upon which they find their insertion. Such muscle fibres have only an indirect relation to the bone since their actual attachment is direct to the periosteum. Such is the case with these bipenniform muscles. Only

through the medium of this tendon-investing, connective tissue sheath, or peritendinum, comparable to the periosteum in the above instance, do the muscle fibres establish their connection with the tendon. The matter involved then is a consideration rather of the relation of the muscle fibrillae to the peritendinum (peritenonium) fibrillae.

A similar instance of a muscle fibre of this type with its attached tendon is represented in fig. 5 under a magnification of 1500 diameters. The peritendinum, consisting of several layers of connective tissue fibres and cells, separates the obliquely-inclined muscle fibre from the tendon. Due to a tear in the tissues an interval exists between a portion of the muscle fibre and the peritendinum and another between the latter and the tendon. In the rest of their extent however, the various structures have maintained their proper relationship to each other. The figure demonstrates five muscle fibrillae (*A*), which proceed directly up to the sarcolemma without losing their features of cross-striation, such as was the case observed with the mouse and chicken extrinsic eye muscles. The tendon end of the sarcolemma is very noticeably thickened and presents upon its internal surface several small elevations upon each of which a muscle fibril is inserted. Sections stained with picro-fuchsin demonstrate that such elevations belong to the sarcolemma rather than to the muscle fibrillae. Moreover, sections stained with hematoxylin-fuchsin indicate that the sarcolemma, with these elevations has a different staining reaction from that of the peritendinum, and that both of these differ from the tendon. Morphologically there is no evidence that the sarcolemma is any other than a homogeneous structure unperforated and untraversed by any formed fibrillae. With differential staining it appears as a thickened homogeneous, unstriated, and non-fibrillar membrane. Nor is there any evidence of the peritendinum fibrillae turning at an angle to perforate it.

Another instance of the general type of muscle termination as demonstrated in figs. 1, 2, and 3 is furnished by the caudal musculature of the tadpole. A portion of a muscle fibre with its attached tendon fibrillae is represented in fig. 6. This fibre was removed from a tadpole about 5,0 cm long. The sarcolemma is very thin and is seen in the figure to be drawn out into a number of cone-shaped processes. Into each of these prolongations as many as from ten to forty muscle fibrillae enter and, without suffering any reduction in diameter or losing their features of cross-striation,

proceed directly up to the internal surface of the sarcolemma with which they fuse. To each one of these cone-shaped sarcolemma processes a single tendon fibril is attached. These fibrillae vary among themselves in diameter, the average size is, however, about that of a muscle fibril, which, on the contrary, are generally uniform in size. Were the muscle fibrillae in direct continuity with the tendon fibrillae then each one of the former must be reduced greatly in diameter before becoming continuous with a tendon fibril. There is, however, no morphological evidence of such a reduction in size.

Unlike the tendon end of the sarcolemma in such muscles as are of the bipenniform type, the sarcolemma of these cone-shaped processes is not noticeably thickened. On the contrary, it is remarkable for its uniform thinness. Were it thickened, one might look therein for morphological evidence of muscle fibrillae, reduced in calibre, passing along its surface or through its substance in order to establish a conjunction with the single tendon fibril.

I have already stated that the tendon fibrillae vary in size. Such variations are not always proportionate, however, to the variations in size of the sarcolemma processes to which they are attached or to the number of muscle fibrillae therein contained. Such might be the case were a direct continuity of the two structures present. The absence of a correlation in size among these several structures might be adduced, therefore, as an additional fact arguing against the continuity of the tendon fibrillae with the muscle fibrillae.

In the figure 6 two cells are demonstrated among the tendon fibrillae. Judging from their morphological appearances and their relation to the tendon fibrillae, I have concluded that such cells were fibroblasts. In some instances I have found the tendon fibrillae traversing the cell protoplasm. In the figure the larger of the two cells gives off two delicate fibrillae each of which is attached to a pointed extremity of the sarcolemma. These are, moreover, the only fibrillae attached to these respective sarcolemma extremities. Upon the other side of the fibroblast several similarly slender fibrils stream off in the general direction of the other tendon fibrillae. I have, naturally, interpreted such fibroblastic processes as developing tendon fibrillae, and upon this interpretation have found an explanation for the disparity in size between the several fibrillae i. e. the smaller tendon fibrillae represent younger fibrillae. Hence the size of such fibrillae is in no direct wise associated with the size

of the cone-shaped sarcolemma processes upon which they are inserted nor with the number of muscle fibrillae which terminate in such sarcolemma processes.

Apart from these considerations, however, the staining reactions demonstrate very clearly that the tendon fibrillae pass only up to the sarcolemma. They do not penetrate it, neither do the muscle fibrillae. The thickness of the sarcolemma everywhere separates the two structures from each other.

Further evidence is contributed by the developing intercostal muscles which I have studied, and of which I have sketched three fibres in figs. 7, 8, and 9. In figure 7 each of the three pointed extremities of the sarcolemma has a connective tissue fibril attached to its apex. The fibril on the right is seen to be derived from a distinct cell body which encloses a relatively large nucleus. The elements of cross-striation do not accompany the muscle fibrillae up to the extremity of the sarcolemma. In the vicinity of the latter these fibrillae somewhat resemble the tendon fibrillae in morphological characters, yet there is no morphological appearance to be noted from which it might be inferred that the homogeneous sarcolemma is perforated by the passage of either form of fibril. The staining of the section shows, in addition, that whereas the muscle fibrillae are thin and very faintly stained, the tendon fibrillae in these instances are relatively deeply stained and much thicker. At no place have I seen these tendon fibrillae inside of the sarcolemma as SCHULTZE has represented in his figures.

Fig. 9 represents somewhat semidiagrammatically the typical muscle termination observed in these developing muscles. The termination of the muscle fibre as a whole is seen to be bluntly pointed, but the sarcolemma end is observed, in addition, to present upon close examination with a high power very many small cone-shaped processes to each of which a connective tissue fibril is attached. The younger the muscle fibre, the smaller are these processes and the less the numbre of muscle fibrillae terminating within each of them. In the course of development as these processes increase in size and the number of contained muscle fibrillae partakes of a proportional increment, but a single tendon fibril, notwithstanding, is found to be attached to the apex of these processes, in other words these apical fibrillae do not multiply in this type of muscle. Herein lies another significant fact denying the continuity of tendon fibrillae with muscle fibrillae.

Fig. 8 is of especial significance because it represents in the same muscle fibre the two forms of muscle ending which I have mentioned above, the one in which the fibre is inserted upon on obliquely-placed surface, as represented by the instance of the bipenniform muscles sketched in figs. 4 and 5, and the other where the insertion takes place upon a tendon extremity whose fibrillae pursue the same linear direction as those of the muscle fibrillae. At the same time this particular fibre affords an explanation of the genesis of the sarcolemma eminences or projections to which I have previously referred and figured in sketches 4 and 10 (*A*), which occur upon the internal surface of the tendon end of the sarcolemma and afford attachment to the muscle fibrillae. The right side of this sketch demonstrates three cone-shaped sarcolemma prolongations to each of which a tendon fibril is attached. These fibrils are typical of the arrangement observed in the tadpole tail. That portion of the sarcolemma end upon the left side of the figure, however, is closely applied to the obliquely-placed fibrillae of the perichondrium enclosing the cartilage of the developing rib. The component structures upon this side of the muscle fibre have the same arrangement as was seen in the bipenniform muscles. The arrangement upon the right side is to be regarded accordingly as transitional, since in the adult animal all of the muscle fibrillae are disposed as is shown upon the left side of the figure. This single muscle fibre, then, represents at once the earlier developmental condition and as well the adult condition; therefore, there can be found in it the probable explanation of the origin of those projections of the sarcolemma which are shown in fig. 10 (*A*).

In the earlier developmental stages the muscle fibres terminate in the manner represented in fig. 9 i. e., by a number of cone-shaped sarcolemma processes to each of which a connective tissue fibril is attached. In the course of development as the muscle fibres gradually approach and are applied to their definitive insertion, in such instances where this insertion is upon an obliquely inclined structure, as periosteum or peritendinum, the sarcolemma loses these cone-shaped terminal features. This takes place by a flattening of the apices of these cones and a synchronous fusion of the adjacent walls of neighboring cones. By the flattening of the terminal sarcolemma a better adhesive surface is presented to the flat surface of the structure affording attachment to the muscle. By the fusion of the adjacent cone-walls is brought about the presence in the

adult of the projections of the sarcolemma which extend into the muscle fibre. They have no connection developmentally or morphologically with the tendon fibrillae, since at first there are no tendon fibrillae attached to that portion of the sarcolemma from which these projections are derived. Neither is it probable that they represent a portion of the muscle fibrillae which might have become transformed into sarcolemma-like tissue.

This genetic sequence explains as well the appearances presented by such muscles as I have represented in the first three figures where the tendon fibrillae and the muscle fibrillae occupy the same linear direction and where in the adult condition the several pointed extremities of the sarcolemma, characteristic of the younger developmental stages, are still demonstrable. The tendon fibrillae occupying the intervals between adjacent extremities assume their position at a stage in the developmental cycle later than the appearance of the definitive form of the muscle fibrillae. In other words, the muscle fibrillae are already attached to the internal surface of the sarcolemma of the intervals before the supplementary tendon fibrillae grow into these intervals and become attached to the external surface of the sarcolemma. Accordingly, we can find another argument in this fact of genesis, in addition to the one based upon morphological grounds and mentioned before, against the acceptance of the view that the tendon fibrillae effect continuity with the muscle fibrillae by perforating the sarcolemma and then coursing a considerable distance in the muscle fibre. Indeed, were structural continuity an established fact, if the chronological order of the development of these »interval« tendon fibrillae and of the muscle fibrillae were alone considered we should expect rather to find a prolongation of a portion of undifferentiated muscle fibrillae through and outside of the sarcolemma in order to meet the developing tendon fibrillae, and not vice-versa as some authors have represented. The presence of extrasarcolemmatous muscle fibrillae has never been observed among vertebrates, so far as I am aware, and in my own preparations is most positively denied by the staining reactions. The only fibrillae lying outside of the sarcolemma are those which have a connective tissue origin.

In general it may be said, then, that the voluntary striped muscles of adult vertebrates terminate in one of two general arrangements the determination of which is dependent upon the relation of the long axis of the tendon to that of the muscle fibre.

When the direction of the two coincide, the muscle fibre of the adult retains its earlier developmental features to the extent that the sarcolemma end still preserves its cone-shaped blunt projections into which the muscle fibrillae, presenting all of their features of cross-striation, enter to fuse with its internal surface. These sarcolemma projections with their intervening recesses are dovetailed into corresponding features of the tendon extremity. The second general type of muscle end is observable in those other muscles where the long axis of the tendon meets that of the muscle fibre at an angle. This form is to be regarded as a developmental derivative of a condition which in the younger specimens conformed to the type of structure which we have designated under I. So far as my own observations have led me I have not yet seen a developing muscle fibre which did not conform to the first type.

In other words, before the muscle fibre has grown far enough to reach its definitive oblique insertion it terminates in cone-shaped sarcolemma processes which give attachment to tendon fibrillae whose direction corresponds to the long axis of the muscle fibre itself.

Those muscles conforming to the bipenniform type of arrangement present the most positive evidence against a continuity of muscle fibrillae with tendon fibrillae, since in these a layer of dense connective tissue, the peritendinum, intervenes and separates the two structures from each other. The features to be considered with especial care in these muscles, therefore, are the peritendinum fibrillae and the muscle fibrillae. Continuity between the latter and the tendon fibrillae is absolutely out of the question. The presence of the greatly thickened sarcolemma end whose homogeneous nature can be very readily made out with a magnification of 1500 diameters, renders possible the most definite answer to the question of continuity so far as these muscles are concerned. In no instance is there the slightest indication of a fibrillar structure perforating this homogeneous membrane. There is surely no turning of the peritendinum fibrillae observable as must be the case were continuity to be established with the obliquely lying muscle fibrillae. We are in a position, therefore, so far as these muscles of the adult are considered, to corroborate most completely the views of Ranvier, Weismann, Kölliker and Motto-Coca, that no continuity exists between the tendon fibrillae and the muscle fibrillae.

The same conclusions may be drawn as well in the instance of those other muscles conforming to this general type where there

are no peritendinum fibrillae intervening between the tendon and the obliquely-placed muscle fibres. Explaining upon developmental grounds the presence of the sarcolemma infoldings into the muscle fibre, and ascertaining through staining reactions the identity of these infoldings in the adult with the sarcolemma, and, in addition, by the same methods most positively establishing the morphological differences between such structures and the muscle and tendon fibrillae, and moreover, failing to observe any turning of the tendon fibrillae at an obtuse angle, as would be necessary to establish continuity with the muscle fibrillae, we are in a position here, as well, to deny in this type of muscle the continuity of tendon fibrillae with muscle fibrillae.

Regarding those other muscles which conform to the first type of termination, where the linear direction of the tendon fibrillae in the adult corresponds to that of the muscle fibrillae, it must be confessed that the problem is not so readily solved, the answer, however, can just as positively be given. The chief difficulty encountered is the exceeding thinness of the sarcolemma and the overlying and underlying of tendon and of muscle fibrillae in vertical optical planes upon the obliquely inclined surfaces of sarcolemma-end processes. The two kinds of fibrillae accordingly lie closer together.

The facts pointing strongly against continuity in this type of muscle are, — each sarcolemma process has attached to it but a single connective-tissue fibril, yet from ten to forty muscle fibrillae terminate in the respective process. A reduction in diameter of each muscle fibril is not demonstrable, such as must be the case if continuity with the single attached tendon fibril existed. Furthermore, there is no indication of a change in direction of the muscle fibrillae at the sarcolemma end in order to join the single apically-attached tendon fibril. The sarcolemma of the process is not thickened as one might expect to find, if the numerous muscle fibrillae turned and passed along its surface or through its substance in order to reach the tendon fibril. Again, the differences in size of the tendon fibrillae are not correlative to the variations in size of the sarcolemma processes or to the number of muscle fibrillae therein terminating. Further, the single tendon fibrillae can be traced up to a connective tissue cell body, a fact suggestive of their genesis. The staining reactions prove that such fibrillae pass only up to the sarcolemma end. In addition, in adult muscles the cross-striation proceeds directly up to the internal surface of the cone-shaped sarcolemma

processes. This fact alone speaks most positively against a perforation of the sarcolemma by the tendon fibrillae and their subsequent passage through the muscle fibre to meet and fuse with the muscle fibrillae. Moreover, there are at first no tendon fibrillae attached to the sarcolemma end in the intervals between the cone-like processes, whereas the muscle fibrillae, undifferentiated in structure are almost from the very first already attached to the internal surface of the sarcolemma at such corresponding intervals.

A word should be said about the combination of fuchsin S with alcoholic-hematoxylin as recommended by several investigators. I have used these stains upon many of my own preparations, with the result that, dependent upon the relative concentration of the former constituent and the time of exposure, I was able to stain with the fuchsin not only the tendon fibrillae but also that portion of the terminal undifferentiated muscle fibrillae lying adjacent to and attached to the sarcolemma end. Indeed, by carrying the staining a little further I was able to stain the neighboring portions of the muscle fibrillae which presented all of the features of cross-striation. Hence, this combination of stains seems to be most unreliable as a criterion of morphological values.

For the developmental and morphological reasons which I have enumerated above, the assertion, that these ends of undifferentiated muscle fibrillae, by reason of their staining reactions, represent tendon fibrillae which have perforated the sarcolemma in order to join the muscle fibrillae, cannot be accepted as convincing to say nothing at all about being most positively denied. Yet some of the published figures intended to represent the continuity of these two kinds of fibrillae and stained with the same stains give exactly this same appearance.

In order to be positive that the cone-shaped extremities of the sarcolemma end were not the result of shrinkage of the tissue in the course of its preparation, I studied specimens of living muscle under high magnifications and ascertained that these processes were characteristic of the termination of the muscles conforming to the first type of ending. I found also that the presence of a uniformly rounded sarcolemma end such as has been figured by other investigators may be accepted as proof positive that the actual end of the muscle fibre has not been represented. I shall have occasion to refer to this feature in connection with fig. 11 upon a subsequent page.

I have presented fig. 10 for the purpose of elucidating certain appearances which have been misinterpreted by various investigators who have endeavored to establish a continuity between muscle and tendon fibrillae. The figure bears, as can be readily noted, a close resemblance to some of their published plates. This figure was sketched from a specimen of thigh muscle of an adult white mouse. Both the tendon fibrillae and the muscle fibrillae pursued the same linear direction. Upon the left side of the fibre the sarcolemma surface (*B*) at the end of the muscle fibre lay in a plane at exactly a right angle to that of the fibrillae, while on the right side it encountered these structures at an acute angle. These two portions of sarcolemma are uninterruptedly continuous with each other (*B—B*). The left side of the fibre demonstrates all of those features to which I have previously referred. The tendon fibrillae are separated from the muscle fibrillae by the thickened sarcolemma end. Upon the right side of the fibre the outline of the sarcolemma because of its obliquity is with much greater difficulty observed. Those fibrillae which occupy the same optical plane, for instanee, the uppermost aspect of the section, can be seen to be separated from each other by the thin cut edge of the sarcolemma. Those other tendon fibrillae which occupy the middle of the thickness of the specimen seem to have perforated that membrane and to have extended into the muscle fibre. This appearance is naturally referable to the fact that they are attached to the under surface of obliquely inclined sarcolemma and hence underlie the uppermost muscle fibrillae. Their intense red stain, derived from the fuchsin, lends its color, too, to these adjacent overlying muscle fibrillae and, consequently, heightens the impression that a portion of them extends into the muscle fibre. A careful consideration of the left side of the sketch is sufficient, however, to eradicate all doubt of the discontinuity of the two kinds of fibrillae.

The particular criticism that I would raise against O. SCHULTZE's work in that he has neglected to explain an appearance which is represented in almost every one of his figures, and which is of fundamental importance in our conception of the relation of the sarcoplasm to the sarcolemma. At places he has represented groups of three, four, and more muscle fibrillae which together perforate the sarcolemma and which are then prolonged as tendon fibrillae, i. e., the sarcolemma is interrupted at the point of perforation of not single muscle fibrillae but of groups of fibrillae. In other words,

the intervening sarcoplasm, as well as the fibrillae, is continued beyond the limits of the sarcolemma end outside of the muscle fibre. Still no attempt is made to explain with what the sarcoplasm becomes continuous or where it ends. Were the condition true, as the author has figured, then we should be compelled to modify our conception of the sarcolemma as a closed tube or envelope confining the semifluid sarcoplasm.

I have studied my own tadpole preparations with this particular point in mind and have represented in fig. 11 a muscle termination which may be considered as typical of the developing fibres at this age. The tadpole measured 1,5 cm.

I agree for the present with other investigators in naming the nucleus, which is seen imbedded in the granular protoplasmic mass surrounding the muscle fibrillae, a myogenetic nucleus, and also in referring to the investing membrane as sarcolemma (B). I have carried this membrane around the muscle fibre end, since such is the appearance produced when focusing down upon the fibre. It does not represent the end of the muscle fibrillae, however, as might at first appear, even in spite of their undifferentiated appearance. This fact can be ascertained by focusing to a deeper level in the fibre, then we get the appearance such as I have represented. The muscle fibrillae terminate in a number of cone-shaped processes, whose walls are formed of a delicate membrane, continuous with the sarcolemma, which bridges the ends of those fibrillae. This appearance is similar of that observed in the muscle fibre represented in fig. 6. These are the processes and this the membrane which have been overlooked. The specific remarks which I have made upon foregoing pages regarding the features to be noted in connection with fig. 6 apply equally well here and require no repetition.

The presence of this unbroken rounded contour of sarcolemma traversing the end of the muscle fibre, to which I have referred above, is readily explained, if we will imagine the muscle fibre as a whole rotated through an arc of 90°. Where the sarcolemma surface faces the eye it is almost perfectly transparent, but where, however, it lies in a vertical optical plane, its contour becomes manifest. Hence, the rounded sarcolemma-end appearance would be demonstrated again in the rotated condition of the fibre by that portion located at A in the figure. At the same time we can readily understand how the muscle fibrillae lying at a deeper level appear to have passed outside of the sarcolemma. And when through

imperfect fixation or staining we overlook the exceedingly delicate, definitive, cone-shaped, sarcolemma processes, the conclusion is natural that the tendon fibrillae and muscle fibrillae are continuous because the sarcolemma has been perforated. This figure answers the question, as well, as to the relation of the sarcoplasm to the sarcolemma. Our earlier conception of the latter as a closed tube enclosing the sarcoplasm is correct. At no place does the sarcoplasm pass through the sarcolemma. At every point this thin envelope separates the specialized, semifluid sarcoplasm from the interstitial fluids, lymph, etc., of the outlying tissues.

I have not been able to carry out my studies on either *Hippocampus* or *Amphioxus*. It would be interesting to explain upon phylogenetic and ontogenetic grounds the differences in the adult morphological condition in these vertebrates from that which I have described above in higher vertebrate muscles.

As a final word upon the question of the cone-shaped processes of sarcolemma I desire to repeat with added emphasis, lest some criticisms regarding faulty fixation or shrinkage be made, that as controls to the fixed and stained preparations I studied many preparations of living muscle by vital staining in monochromatic light. Methylene blue aqueous solutions among others were used and of such a degree of concentration that a control tadpole lived for thirty-six hours in the same fluid which was used to stain the caudal muscles. The preparations which I employed were studied ten minutes after the removal of the muscle. No fixatives or dehydrating agents whatever were used. In every one of these living specimens the cone-shaped sarcolemma processes could be readily found. Therefore, they did not owe their presence to an artefactitious change in the muscle structure.

The following general conclusions, then, regarding these various voluntary striped muscles of the tadpole, white mouse, gray mouse, chicken, frog, and calf may be drawn.

1st — In the manner of termination of muscle fibres two general types may be recognized, one in which the long axes of the tendon and of the muscle fibres coincide, and the second in which they meet at an angle.

2nd — In neither of these two types are the muscle fibrillae in continuity with the tendon fibrillae.

3rd — Developing muscle fibres terminate in a number of cone-

shaped processes of sarcolemma to the apex of which a tendon fibril is attached.

4th — In the adult those muscles conforming to type 1 still preserve the apical processes of their sarcolemma end.

5th — In the adult these processes of sarcolemma are dovetailed into the tendon end.

6th — The sarcolemma at the tendon end of such muscles is not markedly thickened.

7th — The central tendon of bipenniform muscles (type 2) is invested by a connective tissue sheath or peritendinum which consists of connective tissue fibres and cells, and which separates the tendon fibrillae from the muscle fibres.

8th — On muscles, conforming to the second type of structure, the sarcolemma end presents a flat surface which rests directly against the attached structure, be it peritendinum, perichondrium, or periosteum.

9th — This sarcolemma end is considerably thickened and is composed of a homogeneous substance.

10th — It presents a number of sarcolemma projections which project into the substance of the muscle fibre.

11th — These projections are derived from the fused, adjacent walls of the cone-shaped processes of the sarcolemma which were present at an earlier developmental stage of the fibre.

12th — The muscle fibrillae in adult muscles of this second type preserve their features of cross-striation up to the sarcolemma.

13th — This sarcolemma end is not perforated either by the tendon fibrillae, the peritendinum fibrillae, or the muscle fibrillae.

14th — The sarcolemma is not prolonged through the tendon, or over the tendon in either type of muscle.

15th — The sarcoplasm does not at any place in either type of muscle pass through the sarcolemma.

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Explanation of figures.

Plate VII.

Fig. 1. An isolated muscle fibre with its attached tendon of an extrinsic muscle of the eyeball of a three-weeks-old chicken. The dovetailing of the sarcolemma end with the tendon fibrillae is characteristic of muscles belonging to this general type. The muscle fibrillae do not present the features of cross-striation at the muscle end. Magnification 1000 diameters.

Fig. 2. Similar to fig. 1 excepting that the muscle fibril groups are separated from each other by two nuclei. Magnification 1000 diameters.

Fig. 3. A muscle fibre from an extrinsic eye-muscle of a white mouse twenty-two days old. It presents the same general features as the two preceeding figures. Four tendon fibrillae (*A*), are to be noted upon the uppermost aspect of the sarcolemma end. Magnification 1000 diameters.

Fig. 4. A portion of a muscle fibre and tendon of a thigh muscle of an adult white mouse, which demonstrates the kind of muscle ending comprised under group 2. The tendon fibrillae (*A*), are separated from the muscle fibrillae (*C*), by the peritendinum (*B*), which contains four cells. Magnification 1000 diameters.

Fig. 5. Similar to the preceeding. The features of cross-striation can be traced up to the thickened sarcolemma where each is inserted upon a small raised elevation (*B*) of the latter. Magnification 1500 diameters.

Fig. 6. A portion of a muscle fibre of a caudal muscle of a tadpole about 5,0 cm long. Each cone-shaped sarcolemma process has attached to it a tendon fibril. Two of the processes derive fibrillae from a large fibroblastic cell situated among the tendon fibrillae. The original preparation from which this sketch was made demonstrates no thickening of the sarcolemma forming these processes.

Fig. 7. A developing intercostal muscle of a five-day old white mouse presenting three cone-shaped sarcolemma processes, each affording attachment to a connective tissue fibril. The fibril on the right is observed to be directly derived from a cell-body. Magnification 1000 diameters.

Fig. 8. Another developing fibre from the same muscle as the preceeding. In this fibre a more advanced stage of development is seen. Part of the muscle fibre is already attached to the perichondrium of the developing rib-cartilage. Three sarcolemma processes upon the right side of the fibre, however, have not yet reached their definitive attachment, but present connective tissue fibrillae affixed to their apex. Magnification 1000 diameters.

Fig. 9. A semi-diagrammatic sketch of a developing intercostal muscle illustrating the pointed processes of sarcolemma each with its attached tendon fibril. The features of cross-striation of the muscle fibre are wanting at the sarcolemma end. Magnification 1000 diameters.

Fig. 10. A thigh muscle fibre and tendon of an adult white mouse conforming to type 2. Upon the left side of the figure several inturned processes of sarcolemma are represented in the muscle fibre (*A*). Upon this side the sarcolemma is cut exactly transversely. Upon the right side of the figure the sarcolemma (*B*) is encountered obliquely by the section knife, consequently the more superficial of the muscle fibrillae are underlaid by the tendon fibrillae. The sarcolemma separates the two structures, still it appears as if the tendon fibrillae extended among the muscle fibrillae having perforated the sarcolemma. Magnification 1000 diameters.

Fig. 11. This is a portion of a muscle fibre from a caudal muscle of a tadpole 1,5 cm long. The presence of the richly granular protoplasmic mass imbedding a nucleus and surrounding the muscle fibrillae may be taken as an indication that the muscle fibre is in a developmental stage. In the adult state such protoplasmic masses are wanting. The membrane (*B*) investing the mass may be provisionally interpreted as the sarcolemma. The presence of the line continuous with the sarcolemma and carried across the fibre end does not represent the fibre end. I have elucidated this appearance upon a previous page (4). The sarcolemma proper is drawn out into a number of processes in which the muscle fibrillae end and upon whose apex a tendon fibril is inserted. Magnification 1500 diameters.

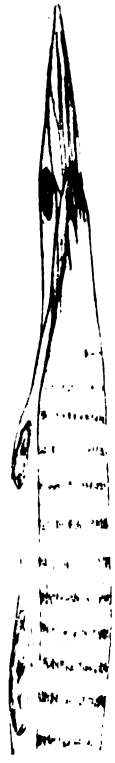


Fig. 1

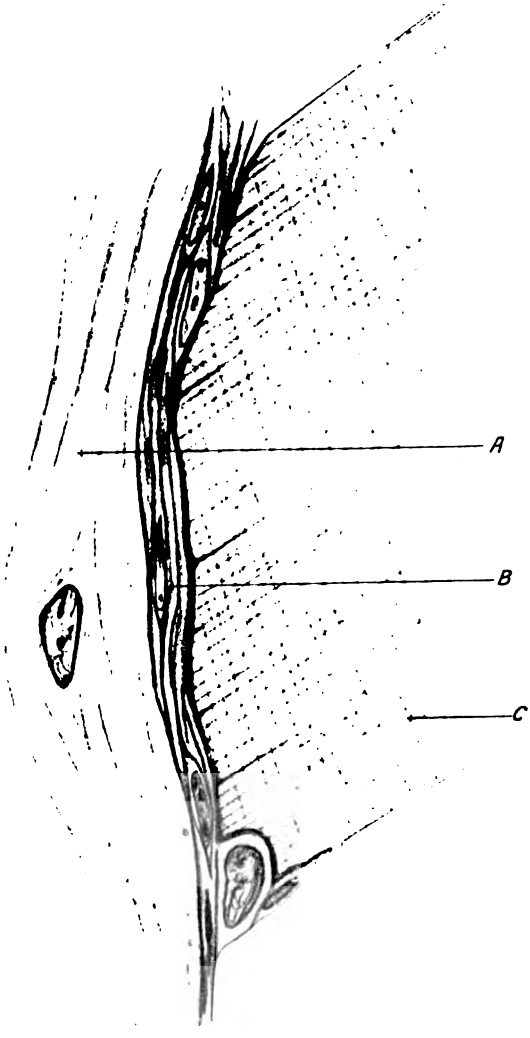


Fig. 4

Fig. 2

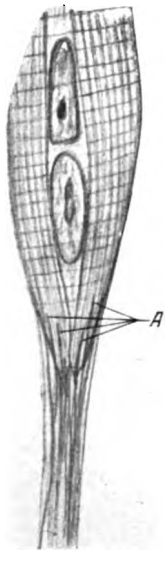
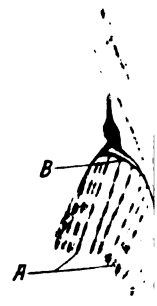


Fig. 3

Fig. 9



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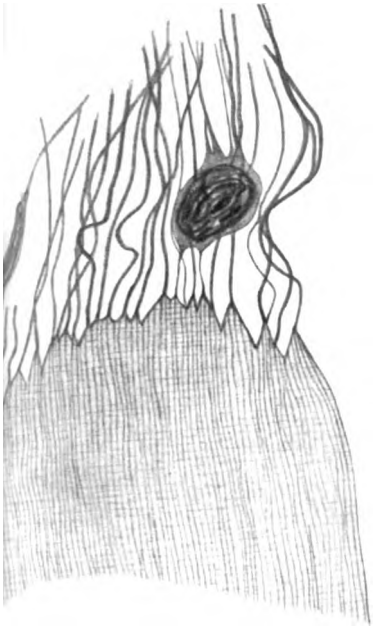

Fig. 6

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Fig. 8

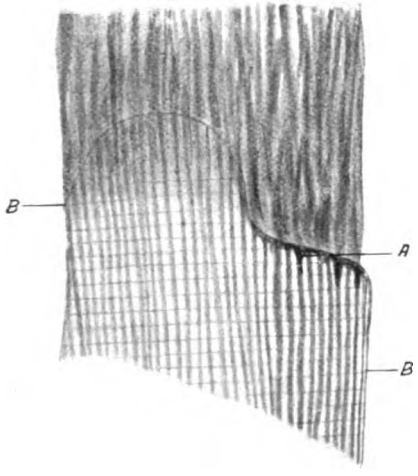

Fig. 10

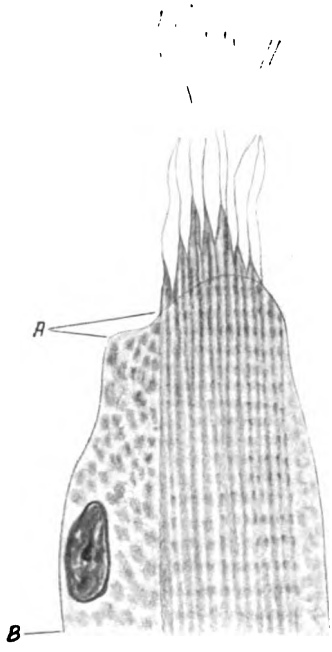

Fig. 11

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Muscle Fibres and Muscle Cells of the adult White Mouse Heart.

W. M. Baldwin.
(Cornell Medical College New York City.)
(From the Biological Laboratory at Bonn.)

With 2 Figures.

In two recent publications[1]) the view was advanced as the result of studies upon various voluntary striped muscles, such as latissimus dorsi, rectus abdominalis, thigh and leg muscles and extrinsic eye muscles of the chicken, cat, calf, white mouse, gray mouse, frog, and caudal muscles of the tadpole, that these muscle fibres of the adult should not be considered, as we have generally hitherto supposed, as large multinucleated cells, but rather as composite contractile structures composed of muscle fibrillae and sarcoplasm and containing muscle cells. That is to say, each one of the numerous nuclei which seem to be immediately imbedded in the sarcoplasm, represents in reality a distinct muscle cell, which presents cellular protoplasm composed of a spongioplasm network with interstices of hyaloplasm and which is completely invested by a cell membrane. This cell membrane intervenes between the protoplasm of the cell, containing the nucleus, and the imbedding sarcoplasm of the muscle fibre. In other words, since these same relations hold which all muscle cells, the muscle fibrillae and sarcoplasm are extra-cellular structures. Hence the generalization usually held, that the adult voluntary striped muscle fibre is a multinucleated cell, as stated before, is erroneous.

Still another fact bespeaking the correctness of these assertions was adduced from the studies of the sarcolemma in similar muscles and detailed in the second communication. It was found in this series that the sarcolemma was a structureless cuticula containing neither cells nor fibrils. Everywhere it stood in direct contact with the sarcoplasm of the fibre and afforded attachment to the telophragmata. Furthermore, its relation to the peripheral-lying muscle cells was such, that it was indented into the fibre by the latter, lying, therefore, between them and the sarcoplasm and not upon the peri-

1) Zeitschrift für Allgem. Physiologie (Max Verworn) 1912.

pheral fibre-aspect of these cells. The one structure found to occupy this position was the cellular and fibrillar investing perimysium of the muscle fibre. Hence such muscle cells lie outside of the sarcolemma. The latter envelope encloses only the highly specialized muscle fibrillae with the semi-fluid sacroplasm. Therefore, for these additional reasons the muscle fibrillae are to be regarded in the adult as extra- or intercellular structures.

An analogy between the histogenetic cycle of the connective tissue group and that of voluntary striped muscle can be drawn. The extremes of the cycle of the latter have been established by a number of competent observers. The intermediate steps, however, require further study. At first the myofibrillae are laid down in the genetic cell bodies. At the opposite end of the genetic course these fibrillae are extracellular. Parallel is the course of development in the connective tissue group; at first appearing as intracellular fibrillae and later being extruded from these genetic cell bodies. These facts obtain as well in the instance of cardiac musculature of the adult white mouse.

In the study of this form of striped muscle fibre the same technic was employed as that detailed in the cited papers. The sections varied in thickness from 2 μ to $3^1/_2$ μ; the stain used was alcoholic hematoxylin.

The first figure represents a longitudinal section of several muscle fibres of the ventricle. The marked morphological structural differences between the protoplasma immediately investing the nuclei and the sarcoplasm of the fibre are apparent at first observation. The cellular spongio-plasmatic network of the former is wanting in the latter. These two forms of protoplasm do not blend with each other; rather, they are sharply delimited from each other by a distinct membrane, the cell wall. With the alcoholic hematoxylin, this structure stained deeply in contrast to the neighbouring parallel-running muscle fibrillae. In focusing down through the section the uninterrupted, membrane-like nature of the cell wall could be readily noted. In contrast to this fact the slender muscle fibrillae pass into and out of focus as their level was reached and passed. Again, the features of cross-striation were not observed on the cell wall, hence the identity of the membrane apart from the muscle fibrillae was established.

The question of the longitudinal extent of such cells, as was the case with similar sections of voluntary striped muscle, is still un-

answered. At the best the solution is exceedingly difficult. The delicacy of the cell wall, the overlying or underlying of it by muscle fibrillae, granulae, and telophragmata, all add to the difficulty in answering the question. The middle of the three muscle cells in fig. 1, however, possibly presents evidence upon which a correct conclusion may be drawn. The uppermost pole of this cell demonstrates what appears to be the reflected edge of the cell wall. The instance is not exceptional, since many such appearances are demonstrable throughout the entire series of sections. But the possibility of its being an obliquely-sectioned cell extremity together with the difficulties enumerated above render, with our present microscopical technic, a positive answer most injudicious.

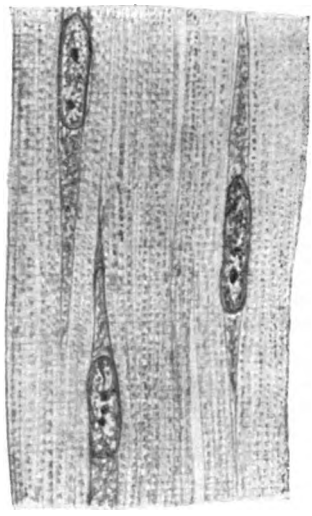

Fig. 1.

Two muscle fibres, represented as transversely sectioned, are seen in figure 2. A blood vessel occupies the angle between them. Each fibre presents a muscle cell. That on the left was encountered at the level of the middle of the nucleus; that on the right above the level of that structure. In the latter the structure of the cell protoplasm is in marked contrast to that of the sarcoplasm. The presence of a cell wall separating the two is unquestionable. The spongioplasm network of the cell is relatively heavily laden with granules. Notwithstanding, the clear fibrillae of this network can in many levels be traced directly up to the internal surface of this cell wall upon which they end. They do not at any place find an insertion upon the muscle fibrillae. A narrow interval of sarcoplasm, equal in general to the cross-diameter of an average muscle fibril, intervenes between the latter and the cell wall.

The cell on the left is of interest chiefly because it demonstrates appearances comparable to those observed in connection with similarly cut sections of voluntary striped muscles. The remarks made in the cited articles regarding such appearances apply here as well. At such levels the membrane appears to be wanting, i. e., the nucleus seems to be immediately imbedded in the sarcoplasm. Hence the

view generally held that the muscle fibrillae are intracellular, being contained in a giant, multinucleated cell. Sections above or below the level of the nucleus, however, demonstrate the cell wall completely circumscribing the cell protoplasm. The inference seems to be justifiable, furthermore, that it is not wanting at the level of the nucleus. In torn preparations where the nucleus has been mechanically removed from its intimate position in relation to the sarcoplasm, and in those other preparations where the nucleus is shrunken, the presence of a distinct wall continuous with the remaining portions of the cell wall can be detected. It appears not to be an artefactitious product, since its outline is regular and definite and it is uniformly and deeply stained. Were it due to retracted and shrunken protoplasm we should expect to find it irregular in outline, varying in thickness, and differing in different levels in intensity of staining. It presents none of these artefactitious criterions. Nor does it in any portion of its extent seem to be merely the free, unthickened edge of the sarcoplasm. It possesses on the contrary a definite contour and a definite staining reaction. Owing merely to the juxtaposition of the nucleus its outline is overlooked in normal unshrunken tissues. Naturally, therefore, at such levels no spongioplasm fibrillae are attached to its internal surface.

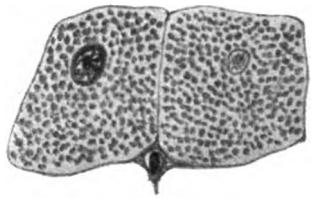

Fig. 2.

The two sketches in figure 1 and 2 are not intended to represent exceptional instances in cardiac muscle structure. Such relations were observed throughout the entire series of cardiac musculature where the conditions were favorable for sharp observation, i. e., where the parts concerned were not obscured by the overlying or underlying by granulae, muscle fibrillae, telophragmata, &c.

In the study of the telophragmata of this type of muscle no instance was observed where these lines traversed either the protoplasm or the nucleus of the muscle cells. This fact may be interpreted as of the following significance. First, it bespeaks the continuity of the cell protoplasm and nucleus as appertaining to a distinct and individual morphological and functional unit, a cell. Furthermore, granting the correctness of the observations of numerous workers that the telophragmata are always directly and uninterruptedly inserted upon the internal surface of the sarcolemma, we should look

for an infolding of the sarcolemma from the periphery of the muscle fibre to invest these cells. No definite proof has been ascertained as yet that such is the instance in the cardiac fibres. The presence of an investment of sarcolemma upon the muscle cells is not, however, negatived by this fact. The telophragmata are attached, apparently, directly to the external surface of the cell membrane. This fact cannot be adduced as conclusive evidence, notwithstanding, arguing against the verified observations mentioned above. The matter demands further observation upon a greater number of vertebrates.

Much remains to be studied upon the intermediate genetic steps of histo-myogenesis. Sufficient evidence of the presence of myofibrillae as intra-cellular structures in the first developmental stages exists in the literature. In the adult stages of both forms of striped muscle, so far as concerns these particular vertebrates investigated, these muscle fibrillae are extracellular. A parallelism can be drawn, therefore, between myogenesis and developmental sequence observed and, seemingly, well established in the case of the connective tissue group of structures. At first the connective tissue and the elastic tissue fibrillae are intracellular. Later in development they are extruded from the genetic cell bodies and occupy, an intercellular position. Such is the sequence as well with the striped muscle fibrillae. In this fact we can find an additional reason, first, for grouping these striped muscle fibres, voluntary and cardiac, among the connective tissue group of structures, and secondly, for not considering them as multi- or singly-nucleated giant cells. In other words the muscle fibrillae and sarcoplasm are inter- or extracellular structures. To this extent these observations corroborate those made upon the voluntary muscles and detailed in the articles cited.

The conclusion arrived at, then, is that our conception of the cardiac muscle fibre as a cell containing fibrillae and sarcoplasm is erroneous as far as concerns the adult white mouse. The terms muscle fibre and muscle cell are not synonymous. The cuticular sarcolemma invests both the highly specialized muscle fibrillae and the sarcoplasm and, in addition, muscle cells. The latter structures present a nucleus, cell protoplasm, consisting of a spongioplasmatic network with interstices of hyaloplasm, and a cell wall. By reason of the last the cells are everywhere excluded from the sarcoplasm and the muscle fibrillae.

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Die Entwicklung der Fasern der Zonula Zinnii im Auge der weissen Maus nach der Geburt.

Von

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Hierzu Tafel XIV und XV.

Der Gegenstand dieser Untersuchung war die Bestimmung des Ursprungs, der Entwicklung und der endgültigen Anordnung der Zonula Zinnii - Fasern des Säugetierauges, indem ich für diesen Zweck eine doppelte Reihe von weissen Mäusen benutzte, im Alter von 12 Stunden bis einschliesslich 17 Tagen, mit anderen im Alter von 22, 27 Tagen und ausgewachsenen Exemplaren. Dazu hatte ich noch Gelegenheit, Exemplare der Katze, des Kalbes und des ausgewachsenen Menschen zu studieren. Die meisten meiner Resultate aber gründen sich auf meine Studien an weissen Mäusen.

Die verwendeten Fixiermittel waren die Flemmingsche Lösung und Sublimat. Die Einbettung geschah nach der Paraffinmethode. Alle Schnitte wurden in einer Richtung gemacht, ausstrahlend vom Mittelpunkte der Linse, und in einer Dicke von 2,5—7,5 μ. Die von mir benutzten Färbemittel waren Safranin, Orcein, Chloralhämatoxylin (Gage) und Bielschowskys Nervenfaserfärbung.

Der erste Teil meiner Arbeit behandelt die Erscheinungen bei 12 Stunden, 5, 11 und 14 Tage alten Exemplaren; im zweiten Teile soll ein fortschreitendes Bild der in der Entwicklung entstehenden Veränderungen gegeben werden, welche die besonderen Gebilde, die in den Bereich unseres Problems fallen, durchzumachen haben, bis sie zu ihrer bleibenden Gestalt und Lage gelangen; im dritten Teile endlich werde ich kurz die Ansichten anderer Forscher, die Entstehung der Fasern betreffend, anführen und nach meinen eigenen Beobachtungen die Gründe für und gegen die Aufrechterhaltung dieser Ansichten ausführlich erörtern.

Die weisse Maus, 12 Stunden alt.

Die Augenlider sind noch nicht geöffnet. Die Netzhaut ist in ihrer Entwicklung so weit vorgeschritten, dass mehrere bestimmte Schichten identifiziert werden können; aber die Schicht der Zapfen und Stäbchen ist noch nicht vorhanden. Der Glaskörperraum ist von einer Menge von Fasern derart durchzogen, dass sie ein Netzwerk bilden, welches viele Zellkörper und Blutgefässe enthält. Dieses Netzwerk reicht, wie man sehen kann, distalwärts[1]) bis zur inneren Oberfläche der Linse und dringt seitwärts von ihr in den Raum zwischen der pars ciliaris retinae und der Linse ein.

Überall, obgleich in der letzten Region weniger bemerkbar, ist auf diesem Netzwerk ein körniger Niederschlag zu beobachten, der sich stark mit Hämatoxylin färbt. Dieser Zustand verdunkelt den feinen Bau der Fasern, der Zellen und der begleitenden Blutgefässe. Die Zellen sind gross, hell und spindel- oder sternförmig und enthalten einen verhältnismässig kleinen Kern. Ihre Ausläufer bilden das körnige Netzwerk. Mehrere solche Zellen sind auf der inneren Oberfläche der limitans retinae interna zu erkennen, längs welcher ihre Fasern zusammen mit anderen, vom Netzwerk ausgehenden laufen. Diese Fasern aber verbinden sich nicht mit der limitans. Die Blutgefässe, in diesem Alter verhältnismässig sehr zahlreich, liegen auf der limitans, oder sie durchziehen das Netzwerk, werden von ihm getragen und erreichen die Linse, auf der sie ein besonders reiches Netz bilden.

Die äussere Oberfläche der limitans stellt eine scharfe Linie gegen das helle Protoplasma der darunter liegenden Netzhautzellen dar, in starkem Gegensatz zu ihrer inneren Oberfläche mit den dazukommenden, aus dem Glaskörpernetzwerk stammenden Fasern. Die limitans selbst erscheint als eine deutliche, dicke und sich dunkel färbende Membran. Bei einigen Schnitten traf ich glücklicherweise ihre flache Oberfläche, so dass ihr Bau gut zu untersuchen war. Es fehlten ihr sowohl Zellumrisse als auch Fasern. Diejenigen Fasern des Glaskörpernetzwerkes, welche sich längs ihrer inneren Oberfläche hinziehen, schienen an keiner

[1]) In dieser ganzen Arbeit habe ich die Ausdrücke „distal“ und „proximal“ angewendet; ersterer bedeutet an dem, oder in der Richtung auf den Hornhautpol des Augapfels hin, letzterer an oder in der Richtung zum Netzhaut- oder Funduspol hin.

Stelle mit ihr zu verschmelzen oder in ihr inneres Gefüge einzudringen. Sie ist durchweg vollständig homogen. Verfolgt man sie distalwärts, so findet man, dass sie auf der Höhe der ora serrata in mehrere Schichten und Fasern sich auflöst, von denen jede sich bis zur Spitze einer ciliaren Epithelzelle der inneren Schicht fortsetzt. Die Linsenhöhle besteht noch; aber die Zellen der durchsichtigen oder proximalen Reihe sind schon in lange Säulen ausgezogen. Die Kapsel erscheint auf beiden Seiten der Linse als eine verhältnismässig dicke fibrilläre Membran, welche ein reiches Netzwerk von anastomosierenden Blutgefässen trägt. Zu bemerken ist, dass die proximale Oberfläche der Linse schon konvexer als die distale ist. Die Hornhaut zeigt drei Schichten, von denen die mittelste viele längliche Zellen mit dunkel sich färbenden Kernen enthält. Die Regenbogenhaut hat sich soweit entwickelt, dass sie nun neben ihrer zweischichtigen Epithellage auf der proximalen Seite eine deutliche Schicht von Mesenchymgewebe zeigt, in welchem an dem pupillaren Rande der Iris die Anlage des m. sphincter iridis zu erkennen ist. Die noch unversehrte Pupillarmembran erstreckt sich durch den pupillaren Zwischenraum, indem sie sich wohl an die distale Linsenkapsel anlehnt, aber nicht mit ihr verschmilzt. Seitwärts kann sie als eine von einer einzelnen Endothelzellenlage gebildete Schicht über die distale Seite des Mesenchymlagers der Iris bis zur Verbindung dieser mit der Hornhaut verfolgt werden. Bei einigen Schnitten ist in dem Raume zwischen dem Irisrande und der Linse ein Blutgefäss zu finden, welches sich bis zur distalen Oberfläche der Linse hinzieht.

Die pars optica retinae setzt sich gegen die pars ciliaris retinae an der ora serrata ab. Schon hat die Entwicklung der Ciliarfalten begonnen, und man kann bemerken, dass sie einen Kern von Mesenchymgewebe einschliessen, welcher Blutkörperchen enthaltende Gefässe besitzt. Das Ciliarepithel ist überall aus zwei Schichten von Zylinderzellen zusammengesetzt. Eine wirkliche limitans ciliaris interna dagegen fehlt. Auf der inneren Epitheloberfläche ist nichts zu sehen als der einfache, dünne Epithelrand dieser scharf gegeneinander abgesetzten Zellen. An dieser Stelle muss noch besonders bemerkt werden, dass die Zellen der inneren Schicht des Ciliarepithels in eine Spitze oder einen Fortsatz auslaufen, und dass durch die ganze Länge der pars ciliaris

retinae jede Zelle einen solchen spitzen Fortsatz besitzt. Solche Fortsätze können tatsächlich distalwärts bis zur inneren Seite der Irisbasis bemerkt werden. Ferner ist zu erwähnen, dass alle jene Epithelzellen zwischen der Spitze der definitiven Ciliarfalten und der ora serrata eine Faser haben, die sich an ihre Spitze ansetzt, dass aber in der Region distal von den erwähnten Teilen viele spitze Zellen zu bemerken sind, welche keinen Faseransatz haben. Von den distalen Verbindungen dieser Fasern und ihrer Bedeutung werde ich später ausführlicher sprechen.

Die limitans retinae interna hört plötzlich an der ora serrata auf. Sie ist nicht distalwärts über das Ciliarepithel verlängert. Die limitans retinae externa ist indessen distalwärts zwischen den beiden Schichten des Ciliarepithels zu verfolgen. Sie ist distalwärts nicht so dick, erscheint jedoch trotzdem als eine deutliche, gleichartige und ununterbrochene lamina.

Das Retziussche Bündel, das sich aus Fasern zusammensetzt, die aus den Epithelzellen unmittelbar distal von der ora serrata hervorgehen und durch den ganzen Glaskörperraum strahlen, wie leicht an Embryoschnitten zu erkennen ist, ist verschwunden und an den Schnitten der 12 Stunden alten Maus nicht mehr aufzufinden. Hier gibt es keine membrana hyaloidea, und demgemäss findet noch keine Trennung des Glaskörperraumes von dem definitiven Zonularaum statt. Viele Blutgefässe mit ihren Blutkörperchen durchziehen diesen letzten Raum. Einige davon setzen sich an die Linsenkapsel an, während andere längs des Ciliarepithels verlaufen. Die grössten dieser Gefässe aber sind in der Mitte des Raumes zu bemerken, getragen von einer dünnen ringförmigen Membran. Diese reicht proximalwärts nicht über die Ebene der ora serrata hinaus und kann distalwärts bis zu jener Stelle der distalen Linsenoberfläche verfolgt werden, wo die Gefässe, welche sie trägt, sich an die Linsenkapsel anlegen. Bei einigen Schnitten liegt sie dicht an der Linse, bei anderen in nächster Nähe des Ciliarepithels. Sie wird in diesen verschiedenen Lagen von einer Reihe von Fasern gestützt, welche an die benachbarten Teile sich anlegen.

Die Membran selbst ist dünn und färbt sich stark mit Hämatoxylin. Bei starker Vergrösserung zeigt es sich, dass sie aus einer oder zwei Schichten von Protoplasmafortsätzen gebildet ist, die aus spindelförmigen oder vierseitigen Zellen stammen.

Diese Zellen, welche in der ringförmigen, stützenden Membran vorkommen, sich gelegentlich aber auch auf den Gefässwänden vorfinden, enthalten einen runden oder ovalen Zellkern und reichlich körniges, sich dunkel färbendes Protoplasma. Gewöhnlich gibt jede Zelle zwei Fortsätze ab, durch deren Vereinigung die hier besprochene Membran gebildet wird. Gelegentlich ist indessen auch nur ein Fortsatz einer spindelförmigen Zelle zu bemerken, welche zur Linse hinübergeht; aber diese Beobachtung ist nur da zu machen, wo die Membran unterbrochen ist. Mitunter sieht man auch einen ähnlichen Fortsatz, der zum Ciliarepithel übergeht, wo er sich an die Spitze einer Epithelzelle anheftet. Solche Fortsätze sind gewöhnlich dick, mit Hämatoxylin dunkel gefärbt und besitzen einen körnigen Niederschlag, ähnlich wie er auf den Fasern des Glaskörperraumes sich findet.

Noch ein anderer Zelltypus kann im Zonularaum nachgewiesen werden. Er ist jedoch anscheinend auf diese Region beschränkt, da ich ähnliche Zellen im Glaskörperraum nicht finden kann. Es sind dies grosse, unregelmässige Zellen mit einem grossen ovalen oder unregelmässigen Kern und reichlichem Protoplasma, welches sich fast gar nicht mit Hämatoxylin färbt. Solche Zellen liegen entweder auf der Gefässhaut oder in dem leeren Raum zwischen ihr und dem Ciliarepithel. Zwischen der Linse und der Membran habe ich diese Zellen nicht gefunden. Jede Zelle ist besonders gekennzeichnet durch die grosse Zahl von Fortsätzen, die sie abgibt. Letztere sind ausserordentlich fein und hell und färben sich nur leicht mit Hämatoxylin. Auch ist auf ihnen kein körniger Niederschlag zu bemerken.

Solche fadenartigen Fortsätze ziehen sich entweder längs der stützenden, ringförmigen Membran oder gegen das Ciliarepithel hin. Dagegen kann ich keine finden, die sich nach der Linse hin erstreckten. Sie verzweigen und vereinigen sich oft, wodurch sie ein dichtes und verworrenes Netzwerk von sehr feinen Fasern bilden, welches zwischen der ora serrata und den Ciliarfortsätzen am dicksten ist. Aber nur wenige Fäserchen dieses Netzwerkes sind distal von dieser Gegend zu verfolgen. Jedoch ist leicht zu beobachten, dass jedes Fäserchen sich schliesslich an die Spitze einer Ciliarepithelzelle festheftet. Solche, die in den Zwischenräumen benachbarter Epithelzellen inserieren, kann ich nicht finden. Die Fasern, welche von den spindelförmigen

Zellen der Gefässmembran entspringen, durchziehen dieses Netzwerk, um an eine Epithelzelle zu inserieren, wie ich früher bemerkt habe; jedoch wegen ihrer Dicke, ihrer dunkeln Färbung und auch wegen des körnigen Niederschlages sind sie leicht von den zarten, hellen, keine Körner führenden Fasern des eigentlichen Netzwerkes zu unterscheiden.

Ein dritter Fasertypus endlich ist noch in dem Raume zu erkennen. Dieser Typus ist indessen nicht oft zu bemerken, sondern ist auf die Spitzen der Ciliarfortsätze beschränkt. Er ist nur bei solchen Exemplaren zu beobachten, wo die Gefässmembran in nächster Nähe jener Gebilde liegt. Es ist ein kurzer und verhältnismässig sehr breiter, schwach körniger Protoplasmafortsatz, welcher von der Ciliarzellenschicht direkt zur Membran geht, mit welcher er sich allem Anscheine nach vereinigt und so einer weiteren Untersuchung sich entzieht. Die Fortsätze dieser Art scheinen einfache Protoplasmafäden der Epithelzellen zu sein.

Die weisse Maus, 5 Tage alt.

Der Glaskörperraum zeigt in diesem Alter weniger Blutgefässe und Zellen, während der körnige Niederschlag auf den Fasern, welche den Raum durchziehen, so gut wie verschwunden ist. Die verschiedenen Schichten der Netzhaut haben sich dem Alter des Tieres entsprechend weiter entwickelt. Die lim. ret. int. endet noch auf der Ebene der ora serrata, indem sie sich in eine Anzahl von Schichten auflöst, welche zu den Epithelzellen gehen. Man kann überdies beobachten, dass jetzt mehr dieser sie zusammensetzenden Schichten vorhanden sind als früher, und dass die Intercellularsubstanz zwischen den Epithelzellen der inneren Ciliarschicht auch dicker ist. Indessen kann man keine Abgrenzung dieser Intercellularsubstanz von dem gleichartigen Bau der Schichten, welche die lim. bilden, erkennen. Die beiden Gebilde stehen in direktem Zusammenhang miteinander. Bei näherer Betrachtung scheinen die verschiedenen Lamellen sich bei der Annäherung an die Fortsätze der Spitze der Epithelzellen zu spalten, und jede Hälfte einer geteilten Lamelle verbindet sich dann sofort mit der benachbarten Intercellularsubstanz. Bei noch weiterem Verfolgen nach der Seite findet man, dass diese Intercellularsubstanz in direkte Verbindung mit der gleichartigen Substanz der lim. ret. ext. tritt und damit verschmilzt. Zu der

Feststellung ihres direkten Zusammenhanges miteinander kommt hinzu, dass diese drei Gebilde: lim. ret. int., Intercellularsubstanz der ora serrata und lim. ret. ext. auch wegen ihres Verhaltens bei der Färbung und wegen ihres morphologischen Aussehens aus derselben homogenen Substanz zu bestehen scheinen.

Die lim. ret. int. geht distalwärts nicht über das Ciliarepithel hinaus. Keine lim. ciliaris interna ist in diesem Alter vorhanden. Die lim. ret. ext. andererseits setzt sich, wie schon früher bemerkt worden ist, zwischen den beiden Schichten des Ciliarepithels ununterbrochen fort. In der Höhe der ora ist sie bemerkenswert dicker als distal dazu.

Die Linsenhöhle ist noch vorhanden. Auf ihrer Kapsel sind anscheinend nicht so viele Blutgefässe zu finden als früher. Die Pupillenmembran ist noch unversehrt.

Der Zonularaum enthält dieselben morphologischen Bestandteile, wie sie in 12 Stunden alten Exemplaren gefunden wurden. Das Netzwerk ist jedoch weniger verworren und die Maschen sind grösser. Die Gefässmembran nimmt dieselbe relative Lage in dem Zonularaum ein, ist aber dünner geworden und weniger deutlich als eine Membran zu erkennen. Sie ist indessen aus denselben spindelförmigen dunkel gefärbten Zellen und ihren Fasern zusammengesetzt wie im vorigen Stadium. Unterbrechungen sind öfters zu bemerken, und in diesen kann man mehrere zarte Fäserchen sehen, die zur Linsenkapsel hinziehen. Diese Fäserchen sind in diesem Alter oft aus den grossen hellen, unregelmässigen Zellen hervorgegangen, welche früher schon beobachtet wurden. Solche Zellen findet man noch in derselben Lage im Zonularaum, nämlich entweder auf der Membran oder in dem freien Raum zwischen ihr und dem Ciliarepithel. Daneben kann man noch beobachten, dass einige der Zellen auf dem Ciliarepithel selbst liegen. Beim Verfolgen der Netzwerkfasern, welche aus diesen unregelmässigen Zellen kommen, bis zum Epithel bemerkt man nunmehr mehrere solche Fasern bis zum Intercellularraum zwischen benachbarten Zellen hinziehen, während bei früheren Exemplaren alle diese Fasern an den spitzen Fortsätzen der Epithelzellen angeheftet waren. Es haben freilich noch nicht viele Fasern ihren apicalen Ansatz verloren; doch ist die Anzahl der Epithelzellen mit Apicalfortsätzen schon merklich verringert. Gelegentlich ist eine dickere Faser, welche aus den spindelförmigen Zellen

der Membran hervorgeht, bis zum Epithel zu verfolgen, wie es schon bei den 12 Stunden alten Exemplaren bemerkt wurde. Diese Fortsätze werden indessen nur selten angetroffen und nur bei Zellen, die in der Nähe der ora serrata und lim. ret. int. liegen.

Die dickeren Protoplasmafortsätze, welche früher ohne Vermittlung eines Zellkörpers direkt vom Epithel zur Gefässmembran liefen, sind, gleichzeitig mit dem Dünnerwerden und dem teilweisen Verschwinden jenes letzteren Gebildes, nicht mehr vorhanden. In diesem Alter ist keine Faser zu beobachten, welche direkt und ohne Unterbrechung vom Ciliarepithel zur Linse geht. Indessen werden viele Fasern gefunden, die, aus einer Zelle des Zonularaumes stammend, direkt zum Epithel gehen, während ähnliche Fasern aus demselben Zellkörper in entgegengesetzter Richtung zur Linsenkapsel laufen.

Die weisse Maus, 11 Tage alt.

In diesem Alter ist die Linsenkapsel von beträchtlicher Dicke, und auf ihr verlaufen viele Blutgefässe. Diese sind auf der proximalen Oberfläche zahlreicher als auf der distalen. Die Linsenhöhle ist infolge der Vereinigung der beiden Epithelschichten, welche ihre Wände bildeten, verschwunden, aber die Pupillarmembran ist noch vorhanden.

Die Ciliarregion hat sich seit dem 5. Tage sehr entwickelt. Die Ciliarfortsätze haben sich bedeutend vergrössert, erreichen jedoch die Linse noch nicht ganz. Jeder derselben enthält einen Kern von Mesenchymgewebe, das Gefässe mit vielen Blutkörperchen umschliesst. Dicht an die äussere Oberfläche der äusseren Schicht der Ciliarepithelzellen setzt sich eine Schicht sich verzweigender verlängerter Mesenchymzellen an. Einige ihrer Fortsätze sind zwischen diesen Epithelzellen zu verfolgen, wo sie sich mit der homogenen Intercellularsubstanz verbinden und nicht weiter verfolgt werden können. In keinem Falle kann man sie durch beide Epithelschichten des Zonularaumes verfolgen.

Die äusseren Ciliarepithelzellen sind kurz, von säulen- oder würfelförmiger Gestalt und körniger als jene der inneren Schicht. Verfolgt man sie proximalwärts, so findet man, dass sie an der ora serrata in die Pigmentschicht der Netzhaut übergehen. Wie bei den jüngeren Exemplaren, kann auch bei diesem die lim. ret. ext. als lim. cil. ext. ununterbrochen distalwärts verfolgt

werden. An der ora serrata ist sie jedoch beträchtlich verdickt. An keiner Stelle ist eine Verschiedenheit ihres Baues von dem homogenen Gefüge der Intercellularsubstanz festzustellen, sowohl bei der äusseren, wie bei der inneren Schicht der Epithelzellen, mit denen sie sich direkt verbindet.

Weiterhin ist zu bemerken, dass die Intercellularsubstanz zwischen jenen inneren Epithelzellen, welche in dem Gürtel zwischen den Ciliarfortsätzen und der ora serrata liegen, und ebenso derjenigen im Raume zwischen benachbarten Fortsätzen gleichmässig verdickt ist. Ein eingehendes Studium des Raumes zwischen diesen Zellen an den auf dieses folgenden Stadien bis zum 14. Tage fortschreitend, zeigt, dass das Dickenwachstum dieser Substanz durch die ganze Länge der Zwischenräume in gleichmässiger Weise stattgefunden hat, d. h. die Zunahme zeigt sich nicht zuerst an einem Ende des Raumes zwischen zwei Zellen und schreitet dann allmählich zum anderen Ende vor. Andererseits zeigt die Substanz zwischen benachbarten Epithelzellen, welche auf den Ciliarfortsätzen liegen, keine so merkliche Zunahme an Dicke.

Ich habe oben erwähnt, dass sowohl die Intercellularsubstanz zwischen den Zellen der äusseren Schicht, wie auch die zwischen den Zellen der inneren Schicht mit der lim. cil. ext. zusammenhängt; jedoch nur an sehr wenigen Stellen liegen diese Intercellularsubstanzen in derselben Ebene und bilden so eine Scheidewand, welche die ganze Dicke des Ciliarepithels durchquert. Der Bau der limitans und der der Intercellularsubstanzen scheint derselbe zu sein: ein zellen- und faserloses, homogenes Abscheidungsprodukt, das sich dunkel und gleichmässig färbt.

Die Zellen der inneren Schicht sind säulenförmig und länger als die der äusseren Schicht. Sie haben ein verhältnismässig helles Protoplasma und einen zentral gelegenen, ovalen oder unregelmässigen Kern. Mitosen sind in beiden Epithelschichten nachzuweisen.

Die inneren Ränder der inneren Epithelzellen, welche sich auf den Ciliarfortsätzen finden, liegen offenbar in derselben Ebene. Eine Anzahl sich dunkel färbender Fasern, die mehr oder weniger eng verbunden erscheinen, liegen auf diesem Epithelrande und sehen wie eine limitans ciliaris interna aus. Man kann indessen an Exemplaren mit gelegentlich kürzerer Epithelzelle, oder wo

infolge der Präparation diese Fasern von der darunter liegenden Epitheloberfläche entfernt sind, leicht erkennen, dass die Ränder dieser Zellen nicht merklich verdickt sind, dass eine wirkliche limitans ciliaris interna in Wirklichkeit nicht vorhanden ist. Ferner erstreckt sich die lim. ret. int. nicht distalwärts über irgend einen Teil des Ciliarepithels hinweg.

Der Zonularaum erscheint in diesem Alter verhältmässig gross und ist vom Glaskörperraum durch die Membrana hyaloidea getrennt. Diese Membran erscheint als ein dünnes, homogenes Gebilde; sie liegt proximal zur Linse und heftet sich an das Epithel der ora serrata ganz ähnlich wie das Ende der lim. ret. int. und unmittelbar distal von ihr. Bei genauer Betrachtung sieht man, dass sie sich in eine Anzahl von Schichten auflöst, von denen jede sich an der Spitze einer Epithelzelle spaltet und sofort mit der Intercellularsubstanz verschmilzt. Auf der distalen Oberfläche der Membran und dicht bei ihrer Befestigungsstelle am Epithel sind viele unregelmässig gestaltete Zellen mit ovalen oder unregelmässigen Kernen und mit mehreren Fortsätzen zu bemerken. Einige derselben sind bis zu den Epithelzellen zu verfolgen, während andere in entgegengesetzter Richtung nach der Linse hin laufen, indem sie längs der distalen Oberfläche der Membran ziehen, mit welcher sie sich schliesslich verbinden.

Einige Blutgefässe sind in dem Raume noch vorhanden. Die Membran, welche sie bei den jüngeren Exemplaren stützte, ist jedoch als eine deutliche morphologische Einheit verschwunden.

Es ist wahr, dass die dunkel gefärbten, spindelförmigen Zellen, welche dieses Gefüge durch ihre vereinigten dicken Fortsätze früher bildeten, noch in diesem Raume vorhanden sind; aber sie sind nur noch auf die Gefässwände beschränkt. Ihre Fortsätze laufen vereinzelt längs der Gefässe und verschmelzen mit anderen ähnlichen Fortsätzen, ohne jedoch eine stützende Membran zu bilden.

Die hellen, ausläuferreichen Zellen der jüngeren Exemplare sind noch ebenso zahlreich wie früher vorhanden und nehmen relativ dieselbe Lage ein wie auf der früheren Stufe. Einige davon scheinen jetzt nur zwei Fortsätze auszusenden, von denen der eine sich zur Linse, der andere zum Epithel hinzieht. Starke Vergrösserung zeigt jedoch, dass dicht beim Epithel jede Faser sich in viele feine Fäserchen teilt, die sich alle entweder an eine Epithelzelle oder an die Intercellularsubstanz zwischen diesen Zellen heften.

Tangentialschnitte belehren darüber, dass viele dieser unregelmässig gestalteten Zellen auf der Ciliarepitheloberfläche liegen, wo sie anastomosierende Fasern abgeben, die das Augeninnere umschliessen. Diese Fasern, sowie auch jene, die ich oben schon erwähnte, und die vom Epithel zur Linse ziehen, nehmen, wenn sie dicht am Epithel der Ciliarfortsätze angeheftet sind, das Aussehen einer limitans dieser Zellen an. Keine der Epithelzellen der Ciliarfortsätze jedoch erhält Fasern. Die letzteren gehen ununterbrochen weiter, ohne mit diesen Zellen in strukturale Verbindung zu treten. Einige der nach der Linse ziehenden Fasern vereinigen sich mit einem Bipolarzellenfortsatz, der auf einer Blutgefässwand liegt; aber keine Faser tritt schliesslich mit den Endothelzellen der Gefässwände selbst in Verbindung.

Ein eingehendes Studium der Anheftungsweise der Fasern an das Ciliarepithel zeigt, dass neben den beiden oben erwähnten Arten der Verbindung, nämlich an die apicalen Fortsätze und an die Intercellularräume der Epithelien noch die folgenden hinzutreten. Manche Fasern, die an den Apicalfortsätzen der Epithelzellen inserieren, haben verschiedene Beziehung zu dem Cuticularrande dieser Zellen und zu der angrenzenden Intercellularsubstanz. In einigen Fällen ist namentlich bei den jüngeren Exemplaren häufiger zu bemerken, dass die zarten Fasern an den Apicalfortsätzen plötzlich aufhören. In anderen sind die Cuticularränder der Epithelfortsätze verdickt und ziehen sich längs der angehefteten Fasern nach der Linse hin. Wiederum kann die Verdickung der Cuticularränder auf eine Seite des Fortsatzes beschränkt sein, während in noch anderen Fällen die Cuticula auf einer Seite des Fortsatzes nur auf eine kurze Strecke der Entfernung von der Intercellularsubstanz bis zur angehefteten Faser verdickt ist. In keinem Falle aber findet man einen verdickten Cuticularrand nur auf die Ansatzstelle der Faser beschränkt und ohne Verbindung mit der benachbarten Intercellularsubstanz. Wo die Fasern in diesem Alter sich direkt mit der Intercellularsubstanz verbinden, sind sie durch diese sich dunkel färbende Masse nicht weiter zu verfolgen.

Die weisse Maus, 14 Tage alt.

In diesem Alter hat das Auge annähernd seine endgültige Beschaffenheit erreicht. Die Augenlider sind offen, und die

Pupillarmembran ist verschwunden. Im Äquator der Linse sind indessen noch einige Blutgefässe zu bemerken. Die membrana hyaloidea, welche den Glaskörperraum vom Zonularaum trennt, ist dicker und dunkler gefärbt, aber ihre Beziehungen und Verbindungen sind dieselben wie bei den Exemplaren von 11 Tagen. Der Zonularaum ist von den zahlreichen Zonulafasern eingenommen, welche gewöhnlich direkt vom Ciliarepithel zur Linsenkapsel ziehen. Sie sind an diese entweder äquatorial, oder wie bei einigen wenigen, distal zu dieser Region angeheftet. Die meisten setzen sich jedoch an jenen Teil der Linsenkapsel an, welcher sich vom Äquator zu dem proximalen Pol erstreckt. Einige der Fasern dieser letzten Gruppe heften sich an die distale Oberfläche der membrana hyaloidea. Man kann diese Fasern eine kurze Strecke auf der Linsenkapsel verfolgen; dann vereinigen sie sich anscheinend mit ihr und sind nicht weiter zu verfolgen. Andere dagegen vereinigen sich mit den Fortsätzen aus den Bipolarzellen, welche sich auf den Gefässen längs der Linsenkapsel befinden.

Die Anheftung der Zonulafasern an die pars ciliaris retinae ist wie bei der 11 Tage alten Maus auf die Teile dieses Epithels, welche zwischen den Ciliarfortsätzen und der ora serrata liegen, und auch auf die Vertiefungen zwischen benachbarten Fortsätzen beschränkt. Keine dieser Fasern ist an dem Epithel auf den Ciliarfortsätzen befestigt. Jede Faser sitzt entweder an der Spitze eines Epithelzellenfortsatzes oder an der Intercellularsubstanz zwischen aneinander grenzenden Zellen an. Es sind jedoch auch verschiedene spitze Epithelzellen zu beobachten, die keine Faseransätze haben. Im ganzen sind weniger spitze Zellen vorhanden als bei den jüngeren Exemplaren. Zu erwähnen wäre noch, dass sie bei den älteren Exemplaren weniger oft auf den distalen Teilen des Ciliarepithels angetroffen werden, aber dass sie selbst im Alter auf den Schnitten niemals ganz fehlen.

Bei eingehendem Studium der Intercellularsubstanz ist leicht zu sehen, dass das, was als direkte Verlängerungen der Zonulafasern erscheint, sich als deutliche faserartige Bänder erkennen lässt, die von der homogenen Intercellularsubstanz, in der sie liegen, sehr verschieden sind, und welche sich dunkel färben. Sie können nach der lim. cil. ext. hin verfolgt werden, ohne diese jedoch ganz zu erreichen. Bei einigen Schnitten, wo das

Messer gerade neben der flachen Oberfläche einer Schicht Intercellularsubstanz eindrang, waren diese Fasern am besten als sich dunkel färbende Fäden zu sehen, die sich nach der äusseren Zellschicht hinziehen. In keinem Falle indessen liegen diese Fäserchen, wie Tangentialschnitte zeigen, innerhalb der Epithelzellkörper.

Nicht alle Zonulafasern gehen ununterbrochen zur Linse, da viele verlängerte, spindelförmige Zellkörper ihren Lauf unterbrechen. Diese Zellen haben einen ovalen oder unregelmässigen Kern, der von reichlichem und in manchen Fällen dunkel gefärbtem Protoplasma umgeben ist. Sie geben in der Regel zwei Fasern ab, von denen eine zur Linse, die andere zum Ciliarepithel geht; hier teilt sich jede in eine Anzahl feiner Fäserchen und erlangt eine Verbindung mit den Epithelzellen ganz ähnlich wie andere Zonulafasern, in deren Verlauf keine Zellen nachzuweisen sind. Zellen sind durch den ganzen Zonularaum zerstreut; sie liegen aber gewöhnlich näher zum Epithel als zur Linse. Einige sind dicht an den Ciliarepithelzellen zu finden und senden Fortsätze aus, die sich mit Fortsätzen aus ähnlich gelegenen Zellen vereinigen und das Innere des Augapfels umkreisen.

Gelegentlich ist auch eine Zelle auf einer Zonulafaser zu bemerken, in welcher nur die Umrisse des Kernes und des Zellkörpers vorhanden sind. Die Faserfortsätze solcher „Schattenzellen“ sind trotzdem gut erhalten und dunkel gefärbt.

Das Ciliarepithel zeigt dieselben Besonderheiten wie bei den 11 Tage alten Mäusen; ausgenommen, dass es sich in verschiedener Hinsicht in einem vorgeschritteneren Entwicklungsstadium befindet. Fortsätze von Mesenchymzellen nahe dem musc. ciliaris treten in die Intercellularsubstanz der äusseren Epithelschicht ein; aber keiner dieser Ausläufer geht hindurch bis in den Zonularaum. Die Intercellularsubstanz der inneren Epithelschicht ist an jenen Stellen, wo Zonulafasern ansetzen, im Vergleiche zu der auf den Ciliarfortsätzen verdickt.

Das Protoplasma mehrerer ciliarer Epithelzellen der äusseren Schicht dringt nach innen zu in den Raum zwischen angrenzenden, darüber liegenden Zellen der inneren Epithelschicht ein. Man findet jedoch nicht, dass die Zonulafasern mit solchen Verlängerungen zusammenhängen. Auf keinem Teile des Ciliarepithels ist eine lim. cil. int. vorhanden. Ich muss auch hier

wieder wie bei den 11 Tage alten Mäusen erwähnen, dass das Aussehen einer solchen limitans dadurch hervorgerufen wird, dass mehrere Zonulafasern sich über die Oberfläche von Zellen auf den Ciliarfortsätzen hinziehen; die lim. ret. int. dagegen erstreckt sich nicht über das Ciliarepithel.

Überblicken wir nun kurz die ganze Reihe der untersuchten Mäuse, so ergibt sich folgendes Bild der aufeinanderfolgenden Entwicklungsstadien:

Vom ersten Tage der Geburt an sind die lim. ret. int. und die lim. ret. ext. als deutliche Membranen vorhanden. Die membrana limitans interna ist indes die stärkere von beiden, und ihre Stärke wird noch dadurch beträchtlich vergrössert, dass sich ihrer inneren Oberfläche zahlreiche Fasern anlegen, die von dem ursprünglichen Glaskörpergewebe herstammen. Diese Membran kann an ihrem äussersten Ende nur bis zur ora serrata verfolgt werden, die schon als die Verbindung zwischen der pars ciliaris retinae und der pars optica retinae bezeichnet wurde. Dort endigt sie, indem sie sich in verschiedene Lamellen teilt, wovon jede sich an eine Epithelzelle der ora serrata anlegt. Dagegen erstreckt sich die lim. ret. ext. von Anfang an an ihrem distalen Ende zwischen die zwei Schichten von Ciliarepithelzellen als eine ununterbrochene Membran, die limitans ciliaris externa. Die nächstfolgenden Tage hindurch nimmt die lim. retinae int. sowohl an Substanz wie auch an Anzahl der Lamellen zu, in die sie sich am äussersten Ende teilt. Die Intercellularsubstanz, mit der die limitans direkt zusammenhängt, wächst in einem entsprechenden Verhältnis an Stärke und ist schon am 6. Tag in merklichen Gegensatz zu der zwischen anderen benachbarten Zellen getreten. Ihr Zusammenhang mit der lim. ret. ext. ist ebenfalls klar ersichtlich, und es muss ferner bemerkt werden, dass letztere an dieser Stelle schon beträchtlich dicker geworden ist.

Schon am ersten Tage sind die Ciliarkörperfortsätze aufgetreten, jeder mit dem zweischichtigen Ciliarepithel und einem Kern aus Mesenchymgewebe, das Blutgefässe mit ihren Blutkörperchen enthält. Die Ciliarfortsätze wachsen allmählich an Grösse und Zahl bis zum 14. Tag, wo sie ihre grösste Entwicklung erreicht haben. Es muss bemerkt werden, dass in den späteren Entwicklungsstufen einige dieser Fortsätze verästelt sind. Ferner beginnt die Intercellularsubstanz, die zwischen ge-

wissen Epithelzellen der inneren Schicht liegt, vom 7. Tage an sich allmählich zu verstärken. Dieses Wachstum findet in einem einheitlichen Verhältnis in der ganzen Länge der Zwischenräume angrenzender Zellen statt. Es muss noch hinzugefügt werden, dass die Teile des Epithels, wo dieses Wachstum stattfindet, sich auf die ringförmige Zone zwischen den Ciliarkörperfortsätzen und der ora serrata, sowie auf die Zwischenräume zwischen den Ciliarfortsätzen beschränken. Gleichzeitig verstärkt sich auch die lim. cil. ext., soweit sie in dieser Gegend anliegt. Eine Unterscheidung der Zellen der äusseren Ciliarschicht von denen der inneren ist von Anfang an möglich wegen ihrer morphologischen Eigentümlichkeiten. Die Zellen der inneren Schicht sind in den jüngsten untersuchten Stadien fast alle spitz, und auf jeder Spitze liegt eine Faser, die von dem den Zonularaum ausfüllenden Netzgewebe herkommt. Mit fortschreitender Entwicklung werden die spitzen Zellen, die auf den Ciliarkörperfortsätzen liegen, allmählich in Zellen umgebildet, die eine flache, gegen die Linse gerichtete Oberfläche darbieten. Diese Veränderung hat sich schon am 5. Tag vollzogen. Die spitzen Zellen sind demgemäss beschränkt auf die zwischen diesen Ciliarkörperfortsätzen liegenden Täler und auf die Zone, die sich zwischen letzteren und der ora serrata ausdehnt. Aber selbst in diesen Gegenden nehmen diese Zellen, indem sie der Verschiebung der Zonulafasern von einer apicalen Insertion zu einer intercellularen sich anpassen, an Zahl ab. Indessen verschwinden sie nie ganz aus einer Schnittserie, da sogar bei der ausgewachsenen Maus noch viele solcher Zellen gefunden werden.

Die Umbildung solcher spitzen Zellen und die Veränderung in der Insertion der Zonulafasern erfolgt gleichzeitig mit dem Wachstum der Intercellularsubstanz, die zwischen den Epithelzellen der Zonulagegend liegt. Zwischen dem 8. und dem 11. Tage kann man diese Veränderungen am besten wahrnehmen. Hinzuzufügen ist, dass durch die ganze Serie Epithelzellen nachgewiesen werden können, die einen spitzen Fortsatz haben, der aber mit keiner Zonulafaser verbunden ist.

Der Glaskörperraum ist am Anfang der Serie mit der entsprechenden Glaskörpersubstanz angefüllt, die aus einem losen Netzwerk von Fäserchen besteht. Diese kommen von verästelten oder bipolaren Zellen her, welche die zahlreichen Blutgefässe umgeben.

Von Anfang an wird auf diesen Zellen und Fasern ein körniger Niederschlag bemerkt. Keine Scheidewand trennt in den früheren Entwicklungsstufen den Glaskörperraum von dem eigentlichen Zonularaum. Die Glaskörpersubstanz wird auch in dem letzteren Raum gefunden und weist dieselben Elemente in ihrer Zusammensetzung und dieselbe allgemeine morphologische Gestalt auf. Es erfolgt sodann eine allmähliche Verminderung in der Zahl dieser Elemente bis zum 5. Tag, wo der Raum von körnigem Niederschlag frei ist. Das Netzwerk ist ebenso in diesem Alter weniger deutlich, aber die Blutgefässe und die sie tragenden Zellen und Fasern sind noch vorhanden und können sogar bis zum 27. Tag nachgewiesen werden, allerdings an Zahl bedeutend verringert.

Die Membrana hyaloidea erscheint nach dem Verschwinden der körnigen Substanz aus dem Zonularaum und hat schon am 10. Tag die morphologischen Eigentümlichkeiten des fertigen Zustandes nahezu erreicht.

Neben den ursprünglichen Bestandteilen der Glaskörpersubstanz, die man im Zonularaum findet, ist dort noch ein Zelltypus vorhanden, der nicht im Glaskörperraum vertreten ist. Es sind dies helle, grosse, schwach gefärbte Zellen mit einem grossen ovalen oder unregelmässigen Kern, reichlichem Protoplasma und einem unregelmässig gestalteten Zellkörper. Zellen von diesem Typus liegen entweder auf der Gefässmembran oder frei im Zonulagebiet. Jede Zelle gibt sehr viel feine, helle Protoplasmafortsätze ab. Diese Fortsätze verzweigen sich, anastomosieren und verschmelzen sehr oft und bilden so ein wirres Netzwerk zwischen der Blutgefässmembran und der ganzen Länge des Ciliarepithels. Die einzelnen Fasern, welche solch ein Netzwerk bilden, heften sich schliesslich an die Spitze einer Ciliarepithelzelle an.

Diese Zellen nehmen an den allmählichen regressiven Veränderungen, welchen die Bestandteile der Glaskörpersubstanz unterworfen sind, nicht teil, sondern bleiben bestehen, und nehmen womöglich mit der fortschreitenden Vergrösserung des Zonularaumes verhältnismässig an Zahl zu. Gleichzeitig aber mit der schnellen Entwicklung der Ciliarfortsätze und mit den übereinstimmenden morphologischen Veränderungen in den Epithelzellen, welche sie bedecken, wird das aus den Zellen hervorgehende

Netzwerk in seiner Lage eingeschränkt. Später heftet es sich nur an das Epithel des Strahlenkranzes und an die Vertiefungen. Zu gleicher Zeit wird es entsprechend den regressiven Veränderungen der Gefässe und der daran gelegenen Bipolarzellenfasern weniger verworren. Auch aus einem anderen Grunde noch anastomosieren, gleichzeitig mit der Vergrösserung des Querdurchmessers des Zonularaumes, die Fasern aus den hellen Zellen viel weniger oft. Sie werden beträchtlich länger und dicker, dunkler gefärbt und verschmelzen in dem Augenblick, wo sie den Zellkörper verlassen. Diese Vereinigung hat die Wirkung, solche helle Zellen bipolar erscheinen zu lassen, wobei eine Faser sich nach der Linse, die andere nach dem Epithel hinzieht. Wenn indessen der letztere Fortsatz bis zum Ciliarepithel verfolgt wird, so stellt sich heraus, dass er sich in eine Anzahl ausserordentlich feiner Fäserchen zerteilt, welche in ihrem morphologischen Charakter und ihrem Verhalten gegenüber der Färbung genau den zarten Fäserchen gleichen, welche das Netzwerk bei den jüngeren Exemplaren bildeten.

Endlich wird das Netzwerk allmählich durch die Zonulafasern ersetzt, welche eine direkte Richtung vom Epithel zur Linse einschlagen. Beinahe jede Faser ist zuerst von einem Zellkörper unterbrochen. Im vorgeschrittenen Stadium vermehrt sich die Zahl der Zonulafasern, welche keine Zellkörper tragen, allmählich. Bei den ausgewachsenen Exemplaren sind sehr wenige solcher Zellkörper zu bemerken. Aber sobald diese verschwinden, treten die „Schattenzellen“ auf. Letztere haben sehr deutliche und anscheinend gut erhaltene Zonulafaserfortsätze, jedoch nur den Umriss eines Kernes und eines Zellkörpers. Ich kann daraus nur schliessen, dass diese „Schattenzellen“ entartete, helle Zellkörper sind, in welchen die Faserfortsätze schliesslich als Zonulafasern des fertigen Auges bestehen bleiben.

Seit dem ersten Bericht von Zinn im Jahre 1775 sind von den Forschern viele und verschiedene Ansichten über den Ursprung, die Bedeutung und die letzten morphologischen Verhältnisse der Zonulafasern aufgestellt worden. Collins (1891) betrachtete sie als Fortsätze der Linsenzellen, welche sich zu den Ciliarepithelzellen erstreckten und schliesslich mit diesen vereinigten, eine Ansicht, die heute von den Gelehrten kaum anerkannt wird.

Eine Anzahl von Forschern haben die Fasern aus dem ursprünglichen Glaskörpergewebe abgeleitet.[1]) Diesen Standpunkt nehmen ein: Lieberkühn, Angelucci, Loewe, Schwalbe, Haensell, Iwanoff, Salzmann, de Waele, Retzius und von Lenhossék. Die Arbeit des zuletzt genannten Forschers wurde auch an Säugetieren, einschliesslich des Menschen, ausgeführt, gründete sich jedoch grösstenteils auf ein Studium von Hühnerembryonen vom 4. Bruttage an. Er sah die Fasern frei in der distalen Verlängerung des Glaskörperraumes zwischen der Linse und dem Ciliarepithel liegen. Sie bildeten zuerst ein verzweigtes Netzwerk, ähnlich dem ursprünglichen, anderswo zu findenden Glaskörpernetzwerk, und lösen sich, unbeeinflusst vom Zusammenhange mit Zellen des ursprünglichen Glaskörpers, der Linse oder des Ciliarepithels, allmählich in deutliche verzweigte Fasern auf, die von der Linse direkt zum Ciliarepithel laufen, wo sie sich zuletzt mit der Intercellularsubstanz jener Region verbinden. Er sah zuerst einen deutlichen Zwischenraum, welcher die Zonulafasern vom Ciliarepithel trennte, der aber später von den Fasern überbrückt wurde.

Lenhosséks Entdeckungen mögen den Ursprung dieser Fasern bei Vögeln zeigen. Meine eigenen Resultate lassen mich jedoch nach dem, was ich gefunden habe, glauben, dass die Entwicklung bei Säugetieren anders verläuft. In diesem Zusammenhang ist es interessant, dass Rabl, der an Menschen, Schafen, Schweinen, Vögeln, Selachiern und Amphibien arbeitete, Ergebnisse berichtete, welche zeigten, dass die Entwicklung der Fasern beim Hühnchen von der bei anderen Tierformen verschieden ist. Bis wir demgemäss den infolge von Schrumpfung der Präparate entstandenen Fehler ausgeschaltet haben, der in einer Anzahl veröffentlichter Zeichnungen zutage tritt, und der den von Lenhossék gesehenen Raum zwischen den Fasern und dem Epithel erklären mag, müssen wir etwas skeptisch sein gegenüber der Fähigkeit der Fasern, sich frei von jeder Zellentätigkeit so zu organisieren, arrangieren und anzusetzen, wie der Verfasser es beschrieben hat. Und wir dürfen nicht den zweiten Fehler begehen, zu folgern, dass das etwa für das Hühnchen Richtige notwendigerweise auch für Säugetiere gelten müsse.

[1]) Auf die Controversen über die erste Entstehung des Glaskörpers wird hier nicht eingegangen.

Zinn, Cloquet, Dessauer, Claeys, Czermak, Topolowski, Collius, Agababow, Terrien und Metzner schlossen, dass die Zonulafasern aus dem Ciliarepithel entstünden. Schoen sah sie für Protoplasmafortsätze an, die aus den inneren Epithelzellen erwüchsen. O. Schultze, Sbordane, Fischel (der am Salamanderauge arbeitete), Rabl und Addario waren derselben Meinung. Damianoff indessen betrachtete diese Fasern als ein Ausscheidungsprodukt dieser Zellen. Schoen bemerkte überdies, dass jede Epithelzelle eine Faser hergab, die durch Verbindung mit ihren Nachbarn eine wirkliche Zonulafaser bildete. Von Spee beobachtete ihren Ansatz an spitze Epithelzellen und schloss daraus, dass sie in Wirklichkeit eine Art von Cuticularprodukt dieser Zellen seien. Salzmann und von Ebner teilten diese Ansicht; letzterer erklärte dazu, er könne sehen, dass einige der Fasern in die Epithelzellen eindrängen. Kölliker behauptete ebenfalls den Epithelstandpunkt die Fasern betreffend, indem er annahm, dass sie in genetischer Beziehung zu den Fasern des Glaskörpers ständen, trotz der tatsächlichen Verschiedenheit ihrer chemischen Reaktionen.

Will man durch Ausschluss zu einem Beweise kommen, auf Grund der oben erwähnten Arbeiten, dass die Zonulafasern wirklich Auswüchse der inneren Ciliarepithelzellen darstellen, so gibt es in bezug auf den Vorgang, durch welchen sie zu ihrer endgültigen Lage gelangen, nur zwei Möglichkeiten.

Die erste ist die, dass die Verbindung zwischen den Epithelzellen und der Linse in einer früheren Periode, als diese Teile einander berührten, entstand, und dass als Resultat des Auftretens des Zonularaumes und der allmählichen Vergrösserung seines Querdurchmessers diese Fasern, welche ihre Ansatzstelle an der Linse noch behaupteten, länger und länger wurden, bis schliesslich der endgültige Zustand erreicht war.

Diese Annahme kann in zwei Hauptpunkten kritisiert werden. Erstens: So viel ich weiss, hat noch kein Autor bei Säugetieren eine Form beschrieben, in welcher selbst in den früheren Stadien die Linse und die Ciliarregion jemals in direkter Berührung miteinander gestanden hätten. Eine Schicht von Mesenchymgewebe mit Blutgefässen trennt diese beiden Gebilde von Anfang an. Zweitens (hier kann ich nur von meinen Beobachtungen an der weissen Maus sprechen): Die von mir be-

merkten spitzen Zellen, welche Protoplasmafortsätze abgeben, die ununterbrochen zur Linse laufen, waren jene auf den Ciliarfortsätzen, wo später keine Zonulafasern angeheftet sind. Drittens: An jenen Flächen, wo die endgültigen Fasern angeheftet sind, habe ich auf den früheren Stufen der Entwicklung keine Protoplasmafortsätze finden können, die direkt und ununterbrochen zur Linse gehen.

Die Zonulafasern müssen sich demgemäss in einer späteren Periode, wenn ein Raum zwischen der Linse und dem Ciliarepithel vorhanden ist, entwickelt haben. Wir können unsere Aufmerksamkeit daher auf die zweite Annahme lenken.

Diese ist kurz folgende: um einen Beweis von dem Epithelursprung der Fasern richtig zu begründen hinsichtlich der unzweifelhaften Tatsache, dass der Raum, durch welchen sie laufen, schon von vielen Mesenchymzellen und Fasern, die selbst einen Epithelansatz haben, eingenommen ist, müssten wir notwendigerweise diese Fortsätze auf verschiedenen Stufen des genetischen Fortschreitens gesehen haben, wie sie aus dem Epithel hervorwachsen und zur Linse fortschreiten, bis sie sich später dort ansetzen. Ein solcher Beweis fehlt in der Arbeit der erwähnten Forscher. Demgegenüber habe ich in meinen Schnitten mehrere Fasern bemerkt, die eine kurze Strecke zur Linse hin liefen und dann plötzlich endeten. Diese Fasern waren immer von anderen begleitet, welche die ganze Strecke zur Linse durchliefen. Selbst mit den besten Linsen, die mir zu Gebote standen, und bei ungefähr 2000facher linearer Vergrösserung habe ich in keinem Falle bestimmen können, ob das Ende der Fasern das spitze Ende einer wachsenden Faser oder das durch das Messer abgeschnittene Ende einer Faser war, welche sich ein wenig unter der Ebene ihrer Nachbarn befand. Auch heben Serienschnitte trotz sorgfältigster Ausführung die Schwierigkeiten nicht auf. Das Haupthindernis ist die Orientierung dieser Fasern beim Übergang von einem Schnitte zum nächsten der Serie. Und wenn man bedenkt, dass sie in einem hellen Raume liegen, verhältnismässig weit entfernt von festen Punkten, die zur Lagebestimmung dienen könnten; wenn man auch die Leichtigkeit bedenkt, wie solche Fortsetzungen durch die Präparation verloren gehen oder verschoben werden, selbst bei den am sorgfältigsten behandelten Exemplaren: so muss man gestehen, dass

hierin eine Schwierigkeit für unser Studium liegt, die bis jetzt fast unüberwindlich ist.

Überdies kann ich bei weiterer Kritik dieser zweiten Annahme hinzufügen, dass meine eigene Arbeit und die vieler anderer Forscher, über deren Ergebnisse ich später ausführlicher reden werde, zeigen, dass die Zonulafasern schliesslich einen Intercellularansatz haben und nicht an einen Apicalfortsatz auf dem Ciliarepithel ansetzen. Gerade wie diese Veränderung des Ansatzes erfolgt, und durch welche verschiedenen Vorgänge sie zustande gekommen ist, das hat keiner der Verfechter des Epithelursprungs erklären können, wenn sie auch den späteren Intercellularansatz ebenfalls bemerkt haben.

Wie können wir weiter das Vorhandensein von Zellen mit deutlichem Zellumriss und Zellkernen auf den Zonulafasern und dem Ciliarepithel erklären bei Mäusen, die beinahe voll ausgewachsen sind? Lenhossék und andere haben runde oder unregelmässige Zellen mit einem deutlichen unregelmässigen Kern in solchen Lagen beobachtet und sie für Leukocyten erklärt. Ich habe diese Zellen ebenfalls gesehen und bin zu demselben Schluss gekommen. Aber die Zellen, auf die ich mich besonders beziehe, geben Zweige ab, die zur Linse und zum Epithel laufen. Wolfrum sah solche Zellen in seinen Exemplaren. Nussbaum beobachtete sie vor ihm beim Kaninchen. Ich habe sie bei jedem Schnitt durch die ganze Reihe der weissen Mäuse gefunden und sie überdies auch im Auge des erwachsenen Menschen bemerkt.

Das Fehlen oder Vorhandensein einer wirklichen Grenzmembran auf dem freien Rande der Ciliarepithelzellen der inneren Schicht ist von mehreren Forschern als Beweis für oder gegen den Lauf der Zonulafasern zu einem tieferen Ansatz in dem Epithel dieser Region angeführt worden. Lenhossék z. B. glaubte an das Vorhandensein einer wirklichen limitans ciliaris interna, deren Funktion es sei, die Zonulafasern mit dem darunterliegenden Intercellulargewebe, mit dem sie direkt zusammenhängt, in Beziehung zu bringen. Diese Membran ist nach ihm ununterbrochen. Darin fand er einen Beweis gegen das tiefere Vordringen der Zonulafasern. Czermak hielt die limitans für eine hyaline Struktur, welche mit dem Glaskörper in direktem Zusammenhange steht. Aus dieser Schicht stammten die Zonulafasern. Topolowsky bestätigt diese Ansicht. Fischel sah die limitans

als die direkte distale Verlängerung der lim. ret. int. an, Salzmann und v. Ebner konnten die Zonulafasern nur bis zur limitans verfolgen. Mawas glaubte an das Vorhandensein einer limitans, stellte aber fest, dass dies kein Hindernis für das tiefere Eindringen der Zonulafasern sei, von denen einige eine Strecke weit in der darunter liegenden Intercellularsubstanz zu verfolgen seien. Der letztere Forscher betrachtete ferner die lim. cil. int. nicht als wirkliche Membran, sondern nur als ein exoplasmatisches Produkt der angrenzenden Zellen.

Ich habe oben von meinen Ergebnissen in bezug auf die lim. cil. int. gesprochen. Bei der Maus ist sie sicher auf keiner Stufe der Entwicklung vorhanden. Indessen erzeugt der Lauf der Zonulafasern und anderer Zellgewebsfasern quer über die Epitheloberflächen an mehreren Stellen das Bild einer Grenzmembran.

Von Claeys wurde ein interessanter Gedanke betreffs einiger analogen und morphologischen Eigenschaften des Ciliarepithels und der eigentlichen Retina 1886 veröffentlicht. Er nahm an, dass dieses zweischichtige Epithel ein System von Stützzellen und -fasern besässe, ähnlich den Müllerschen Fasern der Retina, und dass die Zonulafasern nur die inneren Verlängerungen solcher Fasern wären. Diese Ansicht fand später einen Verfechter in Terrien. Letzterer beschränkte jedoch die Zonulafasern nicht auf diese Stützzellen, da er einige durch die ganze Dicke der Ciliarepithelschichten bis zum äusseren Mesenchymgewebe des Ciliarkörpers verfolgen konnte. Neuerdings unterstützte Metzner diese Theorie und gab an, die Zonulafasern bis zur Scheide des m. ciliaris verfolgt zu haben.

Im Jahre 1906 ging Toufesco so weit, zu behaupten, dass die Zonulafasern aus elastischem Gewebe beständen, dass sie beide Ciliarschichten durchdrängen und so eine direkte Verbindung mit ähnlichem Gewebe in der Aderhaut des Augapfels herstellten.

Ich habe schon früher berichtet, dass ich bei einigen meiner Exemplare Mesenchymzellen bemerken konnte, die an der äusseren Oberfläche der äusseren Epithelzellschicht angeheftet waren, und die gelegentlich Fortsätze abgaben, welche nach innen zu in die Intercellularsubstanz zwischen diesen Zellen eindrangen. Ich konnte sie jedoch nur eine sehr kurze Strecke zwischen diesen Zellen verfolgen, da sie morphologische Eigenschaften und Färb-

barkeit besitzen wie die homogene Substanz, in der sie liegen. Daher bin ich auch nicht imstande, die Beobachtungen dieser Autoren zu bestätigen. Wenn ich nach einigen der veröffentlichten Zeichnungen urteile, kann ich nur schliessen, dass, wie ich bei ähnlichen Erscheinungen unter dem Mikroskop sah, diese Forscher mit Schnitten arbeiteten, die sehr schräg angelegt waren.

Wenden wir unsere Aufmerksamkeit zunächst auf die Intercellularsubstanz, die in den Zonulaflächen des Ciliarepithels vorhanden ist, und welche die von vielen Forschern bemerkten Zonulafaserverlängerungen enthält, so finden wir, dass in Verbindung mit dieser Sache N. van der Stricht, Leboucq und O. van der Stricht, die über die limitans des Gehör-, des Geruchs- und des Sehepithels arbeiteten, zu dem Schlusse gelangten, diese homogenen Membranen seien nicht wirkliche Membranen, sondern nur ein strukturloser intercellularer Kitt. Bei meinen eigenen Serienschnitten kann ich, wie ich schon konstatiert habe, keine geformten Gewebsbestandteile in der limitans bemerken. (Eine Beschränkung dieser Behauptung werde ich später geben.) Ihr morphologisches Aussehen und ihr Verhalten bei der Färbung ist dem der ganzen Intercellularsubstanz des Ciliarepithels, mit der sie in direktem Zusammenhange zu stehen scheint, völlig gleich.

Wenn wir in dem Falle dieser Intercellularsubstanz annehmen, dass sie als eine Art exoplasmatischen Produkts der benachbarten Zellen gebildet ist, wobei eine Zelle nicht mehr als die andere zu ihrer Dicke beiträgt, — und es gibt in der Literatur, wie ich glaube, nichts, was dieser Ansicht widerstreitet — warum sollten wir so weit gehen, zu behaupten, dass diese lim. ext., die allem Anscheine nach aus demselben Stoff zusammengesetzt ist, mehr aus den Zellen der inneren Ciliarschicht hervorgeht, als aus denen der äusseren? Dabei dürfen wir nicht vergessen, dass die Hypothese von der Analogie der inneren Ciliarepithelzellen und derjenigen der Stützzellen der Retina noch nicht sicher gestellt ist. Wir können daher gerechterweise auch nicht vermuten, dass die lim. cil. ext. ein Derivat von Zellkörpern ist, die nach innen von ihr liegen, wie es anscheinend bei der lim. ret. ext. der Fall ist.

Mawas z. B. nimmt den Standpunkt ein, dass die lim. ext. allein von den Epithelzellen der inneren Schicht gebildet ist.

Er gibt indessen in seiner Arbeit nicht genügende Beweise für die Richtigkeit dieser Annahme. Wenn wir dann im Laufe der Entwicklung beobachten, wie die Intercellularsubstanz im Zonulagebiet allmählich an Dicke zunimmt, aber nicht bemerken, dass diese Zunahme zuerst an einem Ende eines Intercellularraumes auftritt und allmählich zum anderen fortschreitet, sondern im Gegenteil in gleichmässiger Weise auf der ganzen Länge des Zwischenraumes vor sich geht: dann haben wir keinen Grund für die Annahme, dass diese Zunahme mehr den Zellen der äusseren als denen der inneren Schicht zu verdanken ist. Wir können daher die Ansicht nicht ganz anerkennen, die von einigen Forschern, z. B. Agagobow, aufrecht erhalten wird, dass die Zonulafasern Ableitungen von den äusseren Epithelzellen seien, obgleich sie aus derselben Substanz wie die Intercellularsubstanz zu bestehen schienen. Und dies auch trotz der Tatsache, dass, wie ich schon früher bemerkt habe, oftmals eine helle Protoplasmaverlängerung äusserer Zellen sich eine kurze Strecke weit zwischen die inneren Epithelzellen einschiebt.

Diese Betrachtungen erklären indessen das faserige Aussehen der Intercellularsubstanz gewisser Regionen nicht, welches von Schultze, Wolfrum, Lenhossék und auch von Mawas bemerkt worden ist. Alle diese Forscher haben das faserige Aussehen mit den Zonulafasern in Verbindung gebracht, indem sie, ausser Lenhossék, annahmen, dass es von den Verlängerungen solcher Fasern herkomme, die in der gleichartigen Intercellularsubstanz eingebettet sind. Sie haben jedoch nicht erwähnt, ob dieses Aussehen auf die Region der Zonulaansätze beschränkt war, oder ob es als charakteristisch bezeichnet werden könnte für alle Intercellularsubstanz durch das ganze Epithel, sowohl zwischen den Zellen der äusseren, wie denen der inneren Schicht. Darin aber liegt ein wichtiger Beweisgrund.

Bei meinen eigenen Untersuchungen habe ich bemerkt, dass diese Faserung vor allem auf die Intercellularsubstanz begrenzt ist, die zwischen jenen Zellen der inneren Epithelschicht lagen, an welche sich Zonulafasern heften. Ich habe sie weder zwischen den Zellen der äusseren, noch zwischen denen der inneren Schicht gefunden, die auf den Ciliarfortsätzen liegen, wo keine Zonulafasern entspringen. Zweitens erscheinen diese Fäserchen zur Zeit der Dickenzunahme dieser Substanz in den erwähnten

Regionen. Drittens ist dieses Aussehen nur in dem Alter zu finden, nachdem die Zonulafasern ihren Ansatz von den Apicalfortsätzen der Epithelzellen in die Intercellularzwischenräume verlegt haben. Endlich sind diese Fäserchen in meinen Präparaten immer in direktem Zusammenhang mit den Zonulafasern zu finden.

Hinsichtlich der Tatsache, dass neuere Forscher die Ansicht mit Nachdruck betonen, dass für das richtige Studium der Zonulafaserverlängerungen in der Intercellularsubstanz besondere Färbemethoden nötig seien, z. B. die „Heldsche Molybdänsäure-Protoplasmafärbung", muss ich nach meiner Erfahrung, — ich habe die Bielschowskymethode angewandt — feststellen, dass die bekannten Färbemittel wie Safranin oder Chloral-Hämatoxylin (Gage) vollständig genügen, um selbst die feinsten Fäserchen zu zeigen. Das einzige Erfordernis für ihre Behandlung besteht darin, die Schnitte sehr stark zu überfärben und dann gründlich zu wässern.

Wolfrums Ansicht, dass mehrere der Zonulafasern die Zellen der inneren Epithelschicht durchzögen, ist nach der Prüfung von Tangentialschnitten des Epithels leicht als ungenau zu erweisen, wie schon Mawas gezeigt hat. Bei meinen eigenen Exemplaren habe ich sicherlich niemals bemerkt, dass eine Zonulafaser eine Epithelzelle durchzog. Ich habe indessen die Zonulafaserverlängerungen nicht durch die Intercellularsubstanz bis zur lim. cil. ext. verfolgen können. Wolfrum jedoch konnte sie bis zu dieser Membran verfolgen, wo sie in kleinen runden Anschwellungen endeten. Die letztere Bildung habe ich ebensowenig auffinden können.

Der zuletzt erwähnte Autor war imstande, bei seinen Säugetierexemplaren Gliazellen zu finden, die von der Retina in der Gegend der ora serrata in den Zonularaum wanderten, wo sie faserähnliche Fortsätze ausschickten, die sich später in Zonulafasern auflösten. Bei meinen eigenen Untersuchungen konnte ich eine solche Wanderung von Neurogliazellen nicht bemerken. Jedoch habe ich aus Wolfrums Beschreibung dieser primitiven Zellen geschlossen, dass die hellen, unregelmässigen, vielverzweigten Zellen, die ich von der ersten Stufe an beobachtete, dieselben sind, die er als Neurogliazellen bezeichnet.

Im Jahre 1895 kam Rochon-Duvigneaud zu dem Schlusse, dass die Zonulafasern „une espèce particulière de fibres

conjonctives" wären. Später entdeckte Nussbaum die Bedeutung solcher Zellen für die Entstehung fertiger Zonulafasern und erklärte sie für Bindegewebszellen und -fasern, die sich sekundär mit der Linsenkapsel und dem Ciliarepithel verbinden. Meine eigenen Schlüsse, die sich auf die Ergebnisse, welche ich in dieser Arbeit niedergelegt habe, gründen, besonders aber der Mangel eines Beweises in meinen Präparaten, die Arbeit Wolfrums bestätigen zu können, lassen mich eine Ansicht annehmen, ähnlich der Nussbaums, dass nämlich die Zonulafasern aus Mesenchymzellen hervorgehen und daher als Mesenchymfasern betrachtet werden sollten.

Die Schlüsse, zu denen ich gelangt bin, sind folgende:

I. Bei der weissen Maus haben die Zonulafasern sich aus Mesenchymzellen entwickelt.

II. Diese Fasern sind zuerst an die Apicalfortsätze der Zellen der inneren Ciliarepithelschicht angeheftet.

III. Später wechseln diese Zonulafasern ihren Ansatz und dringen in die Intercellularsubstanz ein, die zwischen den Zellen der inneren Ciliarepithelschicht liegt.

IV. Im fertigen Auge durchziehen die Zonulafasern die Intercellularsubstanz nach der limitans ciliaris externa hin; aber sie enden plötzlich, ehe sie dieses Gebilde erreichen.

V. Die Zonulafasern endigen nur an jenem Teile des Ciliarepithels, welches in den Tälern zweier benachbarter Ciliarfortsätze und zwischen den Ciliarfortsätzen und der ora serrata liegt.

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Erklärung der Abbildungen auf Tafel XIV und XV.

Fig. 1. Gegend der Zonula im Auge einer 12 Stunden alten weissen Maus. A = distale Partie der Linse; B = ihre Kapsel; C = ein Stück Retina, innen begrenzt von der Mm. limitans interna. Vom Ciliarepithel ist nur der proximale Teil abgebildet, der in Beziehung zur Entwicklung der Zonulafasern (D) steht; E und F = Blutgefässe; G = Stützmembran eines Blutgefässes aus Fortsätzen einer Mesenchymzelle gebildet; den stark gefärbten Zellfortsätzen liegen Körnchen auf. Einige dieser Fortsätze können bis zu den apicalen Fortsätzen der inneren Lage der Ciliarepithelien (H), verfolgt werden. J = grosse, unregelmässig gestaltete und helle Mesenchymzelle mit zahlreichen feinen, hellen Fortsätzen. Dieser Zellentypus ist auf die Gegend der Zonula beschränkt und kommt im Glaskörper nicht vor. Aus solchen Zellen entspringen die Zonulafasern des erwachsenen Tieres. Bei der 12 Stunden alten Maus erreichen, wie Fig. 1 zeigt, die Fortsätze dieser Zellen die Linse noch nicht. Ihre zahlreichen Fibrillen bilden ein dichtes Netzwerk auf dem proximalen Abschnitt des Ciliarepithelium, woran sich die Fibrillen schliesslich festheften. An jedem zugespitzten Fortsatz einer Epithelzelle sitzt eine Fibrille. Diejenigen epithelialen Zellen, welche distal zu dem von den Fortsätzen der hellen Mesenchymzellen gebildeten Netzwerk liegen und späterhin die Ciliarfortsätze decken, verlieren ihre apicalen Fortsätze und sind demgemäss beim Erwachsenen mit der Zonula nicht mehr verbunden. Die Epithelien der ora serrata (K) dagegen und die nächsten distalen liegen im Gebiet der Zonula des Erwachsenen. Es gelang mir festzustellen, dass bei der 12 Stunden alten Maus im Bereich der Zonula die Hauptmasse des fibrillären Netzwerks aus den Fortsätzen der hellen Zellen und nicht von solchen der Epithelzellen gebildet wird. Hervorgehoben zu werden verdient auch, dass die Intercellularsubstanz der Epithelien an der Zonula nicht dicker ist als distal davon, und dass um diese Zeit keine Faser des Netzwerks mit der Intercellularsubstanz der Epithelien verbunden ist. Vergr. 500.

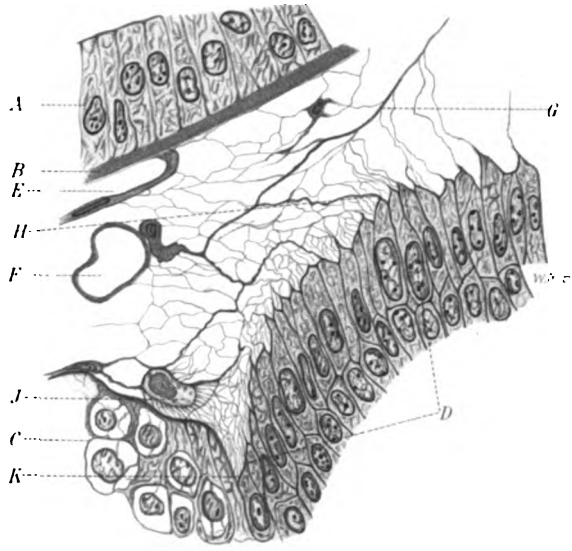
Fig. 2. Aus mehreren Schnitten zusammengesetzte Ansicht der Zonulagegend einer 10 Tage alten weissen Maus. A = distales und B = proximales Epithel eines Teiles der Linse mit ihrer Kapsel C. D = Membrana hyaloidea als eine dünne Membran die Zonulagegend vom Glaskörper trennend und von der proximalen Partie der Linse bis zur ora serrata sich erstreckend (E). Die Retina (F) hat mehrere Zellenlagen und ist innen von der Membr. limitans interna (G) begrenzt. H = Stäbchen und Zapfen, wohl entwickelt. Die Membr. limitans externa der Retina (J) ist distal bis zwischen die beiden Epithellagen des Ciliarkörpers zu verfolgen, wo sie zur Membr. limitans ciliaris externa (K) wird. Die Membr. limitans interna retinae hängt mit der Intercellularsubstanz der Epithelien an der

ora serrata zusammen, direkt proximal von der Anheftung der Membr. hyaloidea. L = ein Stück Iris, M = ein Ciliarfortsatz. Vom Epithel der Ciliarfortsätze entspringen keine Zonulafasern; solche (N), die mit dem Epithel der Zonulagegend zusammenhängen, streichen über die freie Fläche des Epithels der Fortsätze hin. Die Zonulafasern sind halbschematisch eingezeichnet und finden sich am reichlichsten proximal vom Linsenäquator (O). In der Zonulagegend sind zwei Blutgefässe getroffen; das eine enthält im Inneren verschiedene Blutkörperchen und eine Mesenchymzelle (P) auf seiner Wand. Die Fortsätze dieser Mesenchymzelle sind bis an das Ciliarepithel zu verfolgen; viele Zonulafasern gehen in die Intercellularsubstanz des Epithels, distal haben noch verschiedene Epithelzellen zapfenartige Fortsätze. Vergr. 250.

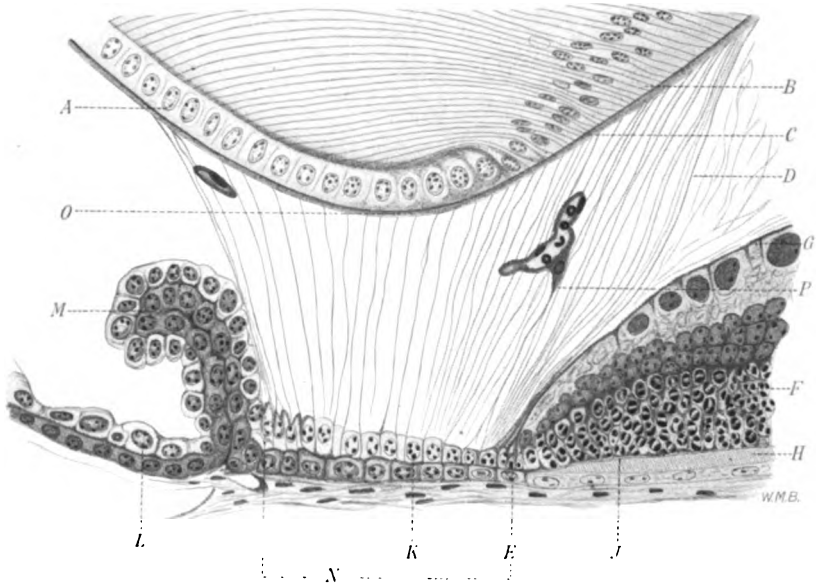
Fig. 3. Nach einem Präparat von einer 14 Tage alten weissen Maus mit offener Lidspalte genau kopiert. A = Retina, B = Ciliarepithel. (Die Retina ist im Schnitt nach vorn verschoben.) C = ora serrata; D = Membr. limitans interna; E = Membrana hyaloidea; die beiden Membranen zerfallen in mehrere Lamellen; jede derselben hängt mit der Intercellularsubstanz der inneren Lage der Ciliarepithelien zusammen. Die Intercellularsubstanz ist an dieser Stelle dicker als sonstwo in der Zonulagegend. F = Membrana limitans externa retinae an der ora serrata verdickt; G = Membrana limitans externa ciliaris mit der vorigen zusammenhängend und die beiden Epithellagen trennend. Eine ächte Membrana limitans interna ciliaris ist an diesem Präparat nicht nachweisbar. Das Epithel der äusseren Lage ist deutlich in Form, Grösse und Granulierung von dem der inneren Lage verschieden. H = Pigmentzellen der Retina. J = Teil eines Ciliarfortsatzes; auf seinem Epithel liegt eine der grossen Mesenchymzellen, wie sie um diese Zeit in grösserer Zahl sich finden. Die Zelle (K) hat zwei Fortsätze; der eine zieht zur Linse und ist kurz abgeschnitten, der andere zerfasert sich auf der Oberfläche des Ciliarfortsatzes. Eine andere grosse Mesenchymzelle (L) liegt im Zonulabezirk auf der Membrana hyaloidea; der eine ihrer verzweigten Fortsätze endet auf der Oberfläche des Epithels der ora serrata. Zwischen diesen beiden Zellen liegen die Zonulafasern; viele derselben gehen in diesem Stadium zwischen die Epithelzellen. Im Vergleich zu dem Stadium von 12 Stunden ist zu bemerken, dass bei dem 14 Tage alten Tier die Zahl der in eine kurze Spitze ausgezogenen Epithelzellen bedeutend abgenommen hat. Zuweilen sieht es aus, als wenn einige Zonulafasern in der Nähe der ora serrata durch Epithelzellen bis zur Membrana limitans externa reichten. Untersucht man solche Stellen aber sorgfältig genug, so stellt sich heraus: dass alle diese Zonulafasern auf der dem Beobachter zugewandten Seite der Zellen in die Intercellularsubstanz eingebettet sind. Keine Zonulafaser geht durch eine Zelle und keine reicht bis an die Membrana limitans externa ciliaris. Vergr. 500.

Fig. 4. Von einer 27 Tage alten weissen Maus. Ciliarepithel zwischen ora serrata (A) und äusserem Rand der Iris (B). C = der mesenchymatische Kern eines Ciliarfortsatzes mit Blutgefäss. Die musivischen Epithelzellen (D) deuten an, dass der Ciliarfortsatz etwas schräg getroffen ist. E = verzweigte Fortsätze tiefer gelegener Mesenchymzellen, die in die Intercellularsubstanz der äusseren ciliaren Epithellage übergehen. An keiner Stelle kann ein Übergang dieser Fasern in Zonulafasern nachgewiesen werden. Die Abbildung zeigt deutlich, dass die Intercellularsubstanz im inneren Zellenlager des Ciliarfortsatzes nicht verdickt ist, im scharfen Gegensatz zum eigentlichen Zonulagebiet nahe der ora serrata. Man findet am Innenrande der Epithelien keine Spitzen mehr wie früher; alle Zonulafasern heften sich an die epitheliale Intercellularsubstanz an. Diejenigen Fasern, welche scheinbar vom Ciliarkörper selbst entspringen, können proximal über den darunter gelegenen epithelialen Rand nach dem Zonulagebiet an der ora serrata verfolgt werden. Diese Fasern erscheinen unter der Form einer Membrana limitans ciliaris interna. Vergr. 500.

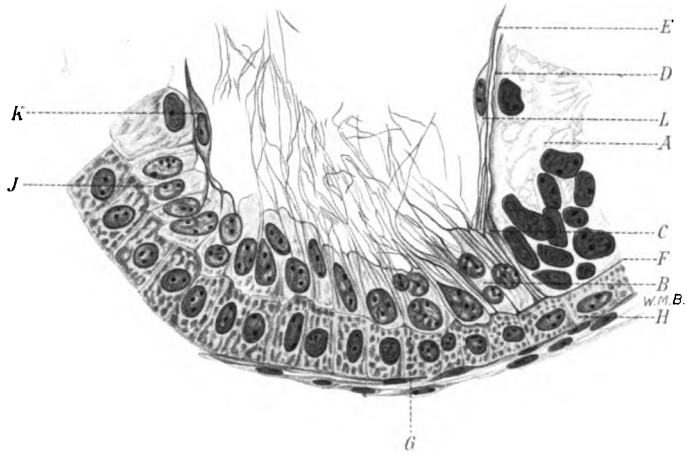
1



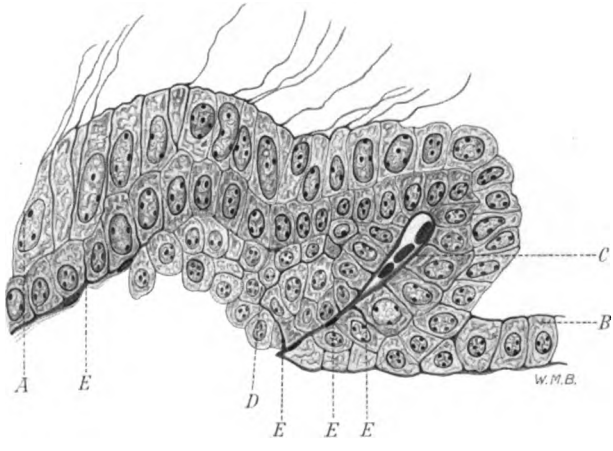
2



3



4



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RHYTHMICAL ACTIVITY OF ISOLATED HEART MUSCLE CELLS IN VITRO

IN previous communications[1,2] I pointed out that the heart muscle of chick embryos will beat rhythmically for many days when suspended in the media of a tissue culture and from such transplanted tissue there is an active growth of cells into the surrounding media. Braus[3] has repeated these experiments, using the hearts of embryo frogs and toads and he has found that these isolated beating hearts react to electrical and chemical stimuli similar to the intact heart. Braus also noted that the cells which grew from the hearts of cold-blooded animals were living at the end of three months. Very recently, Carrel[4] by the use of the method of repeated transplantation of the tissue from a culture to a fresh medium (Carrel and Burrows) has attempted to prolong the life and function of heart muscle *in vitro*. His experiments show that the rhythm which I noted in fragments of embryonic chick hearts can be prolonged, although intermittently, for a period of 85 days. The results of these experiments substantiate, therefore, the former well-known fact, namely, that strips of heart muscle, both of cold and warm blooded animals (Erlanger), will beat for some time when placed in the proper media. In none of these cases could one rule out, however, the possibility of the existence of nerve ganglia or some possible precursor in the young embryonic hearts, which might initiate rhythmical contractions.

During the present year experiments have been made to determine the conditions which would prolong the life and allow the development of functional activity in the cells which had grown and differentiated in the culture. These experiments have shown that the newly grown, cellular syncytia and the isolated single heart muscle cell can become functionally active, beating with a rhythm similar to that of the intact heart.

Pieces of the hearts of chick-embryos of all ages and of young hatched chickens were used. A growth of tissue, composed almost entirely of muscle cells, occurred from all pieces when suspended in the media of both types of cultures, (1) the ordinary hanging drop culture (the plasma modification[1]) of the method of Harrison[5] and (2) a large modified type of culture. This apparatus is so arranged as to supply the tissues continuously with fresh media and to wash away the waste products without in any way disturbing the growing cells. I described this method in detail before the American Association of Anatomists, December 27, 1911.[2] Serum was used as the fluid medium in the latter type of culture.

Rhythmical activity of the newly grown cells was noted in 3 out of 15 of the large type of cultures (No. 2), and in 2 out of 150 of the ordinary hanging drop cultures. These cells were located definitely within the clot and had a clear cytoplasm which contained very few fat droplets. The rhythmical activ-

[1] Burrows, M. T., 1911, *Jour. Exp. Zool.*, Vol. 10, 63.

[2] Burrows, M. T., 1912, *Anat. Record*, Vol. 6, 141.

[3] Braus, H., 1912, *Weiner Med. Wochschr.*, No. 44.

[4] Carrel, A., 1912, *Jour. Exp. Med.*, Vol. XV., 516.

[5] Harrison, R. G., 1907, *Proc. Soc. Exp. Biol. and Med.*, 140; 1910, *Jour. Exp. Zool.*, Vol. 9, 787.

ity did not occur during the active outwandering of the cells but, later, after they became permanently located in a definite portion of the clot and were undergoing slow multiplication and differentiation. In one culture rhythm occurred as early as the fifth day, while in others as late as the fourteenth day of the life of the culture. The greater number of positive results in the large type of culture (No. 2) can be associated with the active and continuous growth of the tissue over a sufficient period of time. Active growth and a regular rhythm has been observed in these cultures for 80 days, while in the hanging drop culture the active growth and the regular rhythm cease after the third or fourth day. The growth then becomes gradually less and the rhythm intermittent, ceasing entirely after 10 or 18 days unless the tissue is transferred to a new medium. The method of repeated transplantation from the culture to a new medium has not as yet been sufficiently developed to allow any increase in the life and the activity of the newly grown cells. At each transfer of the tissue the actively growing and multiplying cells are destroyed and a new growth takes place from those more latently active cells in or about the tissue mass.

The original pieces of heart muscle transplanted to a tissue culture vary as to their rhythmical activity in relation to the portion of the heart from which they are taken as well as the age of the embryo. Pieces of the auricle, especially of that part situated near the entrance of the veins, taken from embryos of all ages and from young hatched chickens, beat when suspended in plasma. The pieces of the ventricle do not beat when taken from embryos older than 10 days, unless special methods of preparation and treatment are used.

Rhythmically beating cells have been grown from the contracting pieces of the hearts of young embryos and from one piece of the ventricle of a fourteen-day chick embryo. The absence of movement in the original mass of tissue of this culture facilitated greatly the study of the delicate contractions of the newly grown cells. The syncytial network which surrounded the original tissue and one isolated cell were beating rhythmically. This cell was situated far out in the clear medium away from all other tissues and beat with a rhythm independent in phase from that of the syncytium. The rate of all beating cells in this culture was the same, 50 to 120 per minute, or a rhythm typical for rhythmical beating pieces of ventricular muscle.

The experiments show: (1) that the cells which have grown and differentiated in a tissue culture can later assume their characteristic function; (2) that rhythmical contraction similar to that observed in the embryonic heart can occur in an isolated and single heart muscle cell; (3) that the rhythmically contracting cells can be grown not only from the pieces of hearts of young embryos, but from the heart muscle of a fourteen-day chick embryo.

These experiments, therefore, give direct evidence for the myogenic theory of the heart beat.

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Rhythmische Kontraktionen der isolierten Herzmuskelzelle ausserhalb des Organismus.

Von Dr. Montrose T. Burrows.

Durch frühere Untersuchungen[1]) ist bewiesen, dass der Herzmuskel des Hühnerembryos, auf geeignete Nährböden implantiert, sich während 8 Tagen rhythmisch bewegt. Dieser Befund wurde von Braus[2]) (Unken- und Froschembryonen) und von Carrel[3]) (Hühnerembryonen) bestätigt. Aus diesen Untersuchungen geht deutlich hervor, dass die funktionelle Tätigkeit des Gewebes ausserhalb des Organismus lange Zeit erhalten bleiben kann. Eigentümlich bei den Gewebekulturen[4]) ist die Auswanderung der Zellen des ursprünglichen Gewebestückes in den umgebenden Nährboden hinein, welcher Vorgang dem eigentlichen Wachstum vorausgeht. Weitere Aufgabe wäre nun zu erforschen, ob die Funktionen solcher ausgewanderten, isoliert liegenden Zellen nach event. Teilung und Differenzierung denen des Muttergewebes gleichwertig sind. Bei der vorliegenden Untersuchung hat es sich tatsächlich herausgestellt, dass die isolierten Herzmuskelzellen rhythmische Bewegungen aus-

[1]) M. T. Burrows: Compt. rend. soc. de biol. 1910, LXIX, 291; Jour. Exper. Zool. 1911, X, 63.

[2]) H. Braus: diese Wochenschrift 1911, S. 2421 u. 2237; Wiener med. Wochenschr. 1911, No. 44.

[3]) A. Carrel, Jour. Exper. Med. 1912, XV, 516.

[4]) Der Ausdruck „Gewebekultur" wird hier in Analogie mit Bakterienkultur gebraucht. Das Prinzip der Gewebekultur besteht darin, dass man ein steril entnommenes Gewebestück auf ebenfalls sterilem Nährboden derart implantiert bezw. suspendiert, dass die Wachstumserscheinungen von Zeit zu Zeit bei Brutofentemperatur verfolgt werden können.

führen. Bei der allgemeinen Bedeutung welche diesem Resultate zukommen dürfte, soll die Methodik, sowie ihre geschichtliche Entwicklung eingehend beschrieben werden. Denn es ist im höchsten Grade wahrscheinlich, dass es auch gelingen wird, bei Anwendung geeigneter Methodik die mehr spezifischen Funktionen anderer hoch differenzierten Gewebe in vitro näher zu verfolgen.

Die erste praktische Anwendung der Gewebekulturmethode, wobei Gewebe längere Zeit ausserhalb des Organismus gehalten und ihre weitere Entwicklung gleichzeitig kontinuierlich beobachtet werden konnte, wurde von Harrison bei seinen Untersuchungen über Nervenfaserentwicklung gemacht. An früheren Untersuchungen über das Ueberleben der Gewebe und Organe hat es freilich nicht gefehlt, aber solche sind meistens von ganz anderen Gesichtspunkten aus unternommen worden. Die Versuche von Wentcher[5]), Ljunggren[6]), Carrel[7]) u. a. haben ergeben, dass es möglich ist, tierische Gewebe lange Zeit ausserhalb des Organismus am Leben zu erhalten. Bei den Untersuchungen von Ranvier[8]), Jolly[9]), Beebe und Ewing[10]) u. a. wurde hauptsächlich auf bestimmte Aeusserungen funktioneller Tätigkeit des in vitro lebenden Gewebe geachtet. Im Jahre 1897 gab Leo Loeb[11]) an, dass es ihm gelungen sei, das Wachstum des Gewebes im geronnenen Blutserum oder Agar ausserhalb des Organismus zu verfolgen. Näheres über die Technik und Resultate gibt er nicht an. Derselbe Autor (1902) beschrieb Versuche über das Wachstum der Haut. Diesmal nahm er Blöcke geronnenen Blutserums resp. Agar, spaltete dieselben, führte das Gewebestückchen dort ein und brachte das Ganze in das Unterhautzellgewebe des Tieres. Weiter untersuchte er bei der Wundheilung, wie das Epithel in den Schorf hineinwuchs.

Die erste Arbeit Harrisons[12]) auf diesem Gebiet stammt aus dem Jahre 1907. Die Methode bestand darin, dass Gewebestücke junger Froschembryonen in einem hängenden Tropfen Froschlymphe suspendiert wurden. Kurze Zeit nach dem Einbringen des Gewebestückes gerann natürlich die Lymphe und bot auf diese Weise ein festes Substrat für das weitere Wachstum des Gewebes. Mit dieser

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[5]) J. Wentcher: Berl. klin. Wochenschr. 1894, 979; Zieglers Beitr., XXIV.

[6]) Ljunggren: D. Zeitschr. f. Chir. 1898, XLVII, 609.

[7]) A. Carrel: Jour. Exper. Med. 1910, XII, 460.

[8]) Ranvier: Traité Technique d'Histologie, Paris 1889.

[9]) Jolly: Compt. rend. soc. de biol. 1903, LV, 1266.

[10]) Beebe und Ewing: British Med. Jour. 1906, II, 1559.

[11]) Leo Loeb: Ueber die Entstehung von Bindegewebe, Leukozyten und roten Blutkörperchen aus Epithel und über eine Methode. isolierte Gewebsteile zu züchten; Chicago 1897, 41. Archiv f. Entwicklungsmechanik d. Organ. 1902, XIII, 487.

[12]) R. G. Harrison: Proc. Soc. Exper. Biol. and Med. 1907, IV, 140; Anat. Record 1908, II, 385; Harvey Lectures, Philadelphia, 1907—1908; Jour. Exper. Zool. 1910, IX, 787.

Methode hat Harrison das Wachstum des embryonalen Zentralnervensystems, des Muskels und der Haut in vitro auf schlagende Weise nachgewiesen. Bei diesen Versuchen wanderten die Zellen längs der Fibrinfäden aus dem ursprünglichen Gewebestück heraus, um sich dort im Fibringerüst weiter zu differenzieren. Aus dem Neuroblastenprotoplasma entwickelte sich selbsttätig der Achsenzylinder. Dadurch wurde die Richtigkeit der His schen Annahme von Harrison eindeutig bewiesen. Gleichzeitig hat Harrison die Grundlage für weitere Versuche über das Wachstum des Gewebes in vitro geschaffen.

Unter Anwendung der von Harrison angegebenen Prinzipien habe ich[13]) im Laboratorium Harrisons die Methode dadurch modifiziert und vereinfacht, dass Blutplasma statt Lymphe zur Verwendung kam. Die Anwendung von Plasma ist deswegen ein wesentlicher Fortschritt, weil man auf diese Weise geeignete Nährböden für das Gewebe verschiedener Tiere leicht gewinnen kann. Dies ist die zurzeit am meisten gebrauchte Methode der Gewebekultur. Neuerdings ist sie von Carrel und mir im Handbuch der biochemischen Arbeitsmethoden Bd. 5, Teil 2, S. 838) ausführlich wiedergegeben worden. Ich untersuchte damals das Wachstum embryonalen Gewebes vom Frosch und Huhn im Frosch- resp. Hühnerplasma und konnte die Resultate Harrisons bestätigen. Ich konnte ferner Kernteilungsfiguren häufig nachweisen. Noch wichtiger erschien mir die Tatsache, dass die funktionelle Tätigkeit des Gewebes in solchen Präparaten lange Zeit erhalten blieb. Beispielsweise führte das Herz von 60 Stunden alten Hühnerembryonen rhythmische Kontraktionen bis zum achten Tage nach der Herstellung des Präparates aus. Wegen der prinzipiellen Bedeutung dieser Tatsachen haben Carrel und ich[14]) mit Hilfe der Plasmamethode den naheliegenden Gedanken verfolgt und das Wachstum der Organ- und Gewebestücke verschiedener Tiere sowie deren Embryonen untersucht. Bei diesen Versuchen gelang zum ersten Male die Kultivierung von Organen und Gewebestücken erwachsener Tiere. Das Wachstum zahlreicher bösartiger Geschwülste (auch von Menschen) in vitro wurde gleichfalls studiert. Kurz darnach haben Lambert und Hanes[15]) ähnliche Versuche mit den Mäuse- und Rattentumoren angestellt. Dabei haben sie die wichtige Beobachtung gemacht, dass diese Geschwulst- und Gewebestücke auch in artfremdem Plasma wachsen. M. R. und W. H. Lewis[16]) haben das Wachstum embryonalen Hühnergewebes in verschiedenen flüssigen und festen Nährböden (Agar) untersucht. Sie konnten als erste das Wachstum dieses Gewebes in einfachen Salzlösungen konstatieren. In seiner ersten Mitteilung hat Harrison auf die Notwendigkeit eines festen

[13]) M. T. Burrows: loc. cit.; Journ. Am. Med. Ass. 1910, LV, 2057.

[14]) Carrel und Burrows: Journ. Am. Med. Assn. 1910, LV, 1379, 1554, 1732; Compt. rend. soc. de biol. 1910, LXIX, 293, 298, 299, 332; Jour. Exper. Med. 1911, XIII, 387, 571.

[15]) Lambert und Hanes: Journ. Am. Med. Assn. 1911, LVI, 33, 791; Jour. Exper. Med. 1911, XIII, 495; XIV, 129, 453.

[16]) M. R. und W. H. Lewis: Johns Hopkins Hospital Bull. 1911, XXII, 241; Anat. Record 1911, V, 277; VI, 207.

Substrats, z. B. Fibringerüst, geronnener Lymphe für die Entwicklung von Nervenfasern und Zellen schon hingewiesen. Als Bestätigung dieser Anschauung konnte ich[17]) Form und Gestaltsstörungen, wie z. B. Abrundungen und Verkleinerungen von Spindelzellen, die von Fibrinfäden losgelockert wurden, beobachten. Wenn solche Zellen wieder in Berührung mit einem festen Körper kommen, strecken sie sich alsdann zu einer länglichen oder unregelmässigen Gestalt aus. Dieser Befund wurde später von Carrel und mir an Tumorzellen bestätigt. Welche Gestalt schliesslich angenommen wird, ist von der Art des festen Substrates abhängig. Harrison[18]) hat nachträglich gezeigt, dass die Zellen, welche sich bei Anwendung eines flüssigen Nährbodens, wie bei den Versuchen von M. R. und W. H. Lewis, an dem Wachstum beteiligen, sich dem Deckglas stets anfügen. Es konnte ferner gezeigt werden, dass Spinngewebe (Harrison), baumwollene resp. seidene Fäden[19]) genügende Stütze für ein gerichtetes Wachstum in flüssigen Nährböden darbieten.

Carrel und ich[20]) haben auf die Möglichkeit sukzessiver Transplantationen von Gewebezellen aus Kulturen auf frischen Nährboden hingewiesen; sekundäre und tertiäre Kulturen erhielten wir leicht. Gelegentlich ihrer Versuche über Krebsimmunität haben Lambert und Hanes[21]) von diesem Prinzip Anwendung gemacht und konnten dabei die Zellen der Subkulturen genügend lang am Leben erhalten, um die Wachstumserscheinungen der Zellen des ursprünglichen Gewebes in einer aufeinanderfolgenden Reihe von Subkulturen auf Plasma verschiedener Herkunft zu untersuchen. Mittelst sukzessiver Transplantation hat Carrel[22]) nachher Gewebe lange Zeit hindurch am Leben erhalten können.

Gerade in der letzten Zeit ist die Plasmamethode auf die verschiedenen biologischen Probleme vielfach angewendet worden. Hier sei besonders auf die Arbeiten von Ruth[23]), Lambert[24]), Carrel und Ingebrigtsen[25]), Loeb

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[17]) M. T. Burrows: loc. cit.

[18]) R. G. Harrison: Science 1911, XXXIV; Anat. Record 1912, VI, 181.

[19]) Carrel und Burrows: Jour. Exper. Med. 1911, XIV, 244.

[20]) Carrel und Burrows: Compt. rend. soc. de biol. 1910, LXIX, 329, 365; Jour. Exper. Med. 1911, XIII, 416.

[21]) Lambert und Hanes: Jour. Am. Med. Assn. 1911, LVI, 587; Jour. Exper. Med. XIII, 505.

[22]) Carrel: Jour. Am. Med. Assn. 1911, LVII, 1611; und 1912, loc. cit.

[23]) E. S. Ruth: Cicatrization of Wounds in vitro. Jour. Exper. Med. 1911, XIII, 422.

[24]) R. A. Lambert: The Production of Foreign Body Giant Cells in vitro. Jour. Exper. Med. 1912, XV, 510.

[25]) Carrel und Ingebrigtsen: The Production of Antibodies by Tissues living outside the Organismus. Jour. Exper. Med. 1912, XV, 287. — Carrel: Berl. klin. Wochenschr. 1912, No. 12; Jour. Exper. Med. 1912.

und Fleisher[26]), Hada[27]), Oppel[28]), Braus[29]) und Weil[30]) hingewiesen. Es sei bei dieser Gelegenheit darauf aufmerksam gemacht, dass in den Arbeiten von Hada und von Oppel die Plasmamethode zur Kultivierung der Gewebe wiederholt als die „Carrelsche Methode" bezeichnet wird. Aus dem Vorhergehenden ist aber ersichtlich, dass die wesentlichsten Fortschritte auf dem Gebiete des Gewebezellenlebens ausserhalb des Organismus fast ausschliesslich mit der Plasmamethode gewonnen worden sind. Auch ist die Plasmamethode nur eine Modifikation der Harrisonschen Methode, und die Modifikation, namentlich die Anwendung von Blutplasma statt Lymphe, ist von mir im Laboratorium Harrisons ausgearbeitet worden. Somit war Carrel auf keine Weise an der Entwicklung dieser Methodik beteiligt.

Methodik: Es wurden Präparate nach zwei Methoden angefertigt: 1. nach der Methode des hängenden Plasmatropfens (Harrison, Burrows), 2. nach einer von mir kürzlich ausgearbeiteten Methode, bei der das Gewebe fortwährend mit frischem Serum umspült wird. Beim zweiten Verfahren wird eine bessere Annäherung an die Verhältnisse im lebenden Organismus erzielt, indem dafür gesorgt wird, dass die Nahrungsstoffe kontinuierlich erneuert werden, während die Bestandteile des zellulären Stoffwechsels gleichzeitig entfernt werden. Denn es ist ohne weiteres klar, dass selbst bei Anwendung des geeignetsten Nährbodens eine Aenderung der Wachstumsgeschwindigkeit auftritt, wenn eine Anhäufung der Bestandteile des zellulären Stoffwechsels stattgefunden hat. Mit Rücksicht auf diese Faktoren habe ich diese Methode ersonnen, in der Hoffnung, dass man dadurch die Dauer des Wachstums verlängern kann, um Aufschluss über den Stoffwechsel sowie andere Aeusserungen funktioneller Tätigkeit zu gewinnen.

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[26]) Loeb und Fleisher: Ueber die Bedeutung des Sauerstoffs für das Wachstum der Gewebe von Säugetieren. Bioch. Zeitschrift 1911, XXXVI, 98.

[27]) S. Hada: Die Kultur lebender Körperzellen. Berl. klin. Wochenschr. 1912, No. 1, 11.

[28]) A. Oppel: Ueber die Kultur von Säugetiergewebe ausserhalb des Organismus. Anatomischer Anzeiger 1912, XL, 464. Archiv f. Entwicklungsmechanik d. Organ. 1912, XXXIV, 132.

[29]) H. Braus: loc. cit.

[30]) Weil: Some Observations on the Cultivation of Tissues in vitro. The Jour. Med. Research 1912, XXVI, 159.

Es sei hier an das wesentlichste der Methodik erinnert[31]) (vergl. hierzu Fig. 1). Mittelst eines Dochtes wird das Medium von einem Behälter (a) durch die Kulturkammer (b) geleitet, um dann in einen anderen Behälter (c) aufgenommen zu werden. In der Kulturkammer wird der Docht in seine einzelnen Fasern, welche sich an der Oberfläche des Deckgläschens festsetzen und ein Kapillarnetz bilden, zerzupft. Das Gewebe wird in kleine Stücke zerschnitten und in das offene Netz von Baumwollfasern gelegt und hier durch das Gerinnen der hinzugefügten Plasmatropfen festgehalten. Der flüssige Nährboden bewegt sich langsam am Docht entlang durch die Kultur und sammelt sich in der Aufnahmekammer (c). Die Nährflüssigkeit an den Geweben wird auf diese Weise fortwährend verändert, ohne dass die wachsenden Zellen in irgend einer Weise gestört werden.

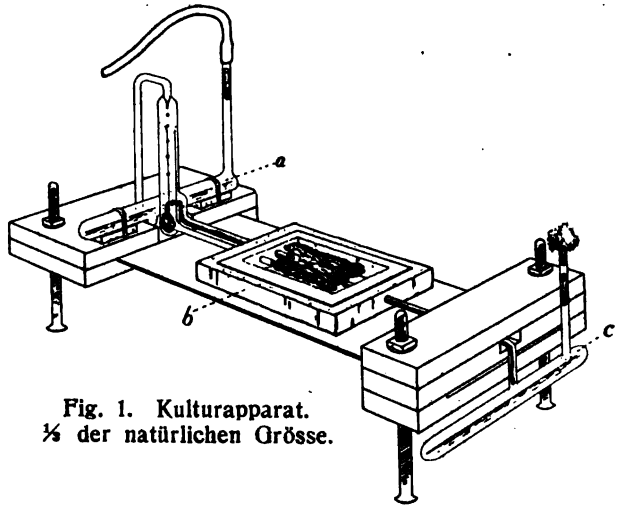

Fig. 1. Kulturapparat.
⅓ der natürlichen Grösse.

Die Zufuhrkammer (a) ist aus Glas gemacht und besitzt zwei Abteilungen. Die Lösung wird von dem horizontalen Behälter nach oben in die vertikalen Abteilungen am oberen Ende des Dochtes geführt. Hierdurch wird eine genaue Regulierung der pro Zeiteinheit benutzten Menge ermöglicht. Serum wurde als flüssiger Nährboden in dieser Reihe von Experimenten benutzt.

Herzen von Hühnerembryonen zu allen Zeiten des embryonalen Lebens, sowie auch die von ausgebrüteten Hühnchen wurden zu den Kulturversuchen verwendet. Von den jungen Embryonen wurde meist das ganze Herz, von den älteren sowie öfter auch von den jungen nur ein heraus-

[31]) Vorgetragen in der Sitzung der American Association of Anatomists am 27. Dezember 1911. Anatomical Record, VI, 141.

geschnittenes Stückchen Herzmuskel auf den Nährboden gebracht.

Das ganze Herz der 60—96 stündigen Embryonen schlägt rhythmisch in Kultur mit einer Frequenz von 50 bis 120 Schlägen in der Minute.

Ob die herausgeschnittenen Stücke pulsieren oder nicht, ist sowohl vom betreffenden Teile des Herzens, welchem sie entnommen werden, als auch vom Alter des Embryo abhängig. Ventrikelstücke von 60 stündigen bis 10 tägigen Embryonen, Stücke des Vorhofs, insbesondere aus der Nähe der Venen der Embryonen beliebigen Alters und des jungen Hühnchens zeigten rhythmische Kontraktionen. Die Ventrikelstücke von älteren Embryonen haben nicht rhythmisch pulsiert [32]). Die Frequenz des Rhythmus ist beim Vorhof grösser als beim Ventrikel; im ersten Falle beträgt sie 150 bis 220, während im zweiten Falle nur 50 bis 150 Schläge in der Minute. Der Rhythmus bei den gewöhnlichen Hängetropfenkulturen bleibt bis zum 3. oder 4. Tage regelmässig und wird später unregelmässig. Dieser intermittierende Rhythmus kann in solchen Präparaten bis zum 17. Tage dauern. In den grösseren Kulturen mit beständiger Zu- und Abfuhr frischen Serums bleibt der Rhythmus regelmässig, sogar bis zum 30. Tage.

W a c h s t u m. Die Wachstumserscheinungen lassen sich in zwei Perioden zerlegen, erstens die der lebhaften Auswanderung der Zellen des ursprünglichen Gewebestückes in den es umgebenden Nährboden hinein; zweitens die der Teilung und Differenzierung. Die erste Periode fängt gegen Ende des ersten Tages an und dauert vom 5. Tage bis in die 2. Woche bei beiden Arten der Kulturen. Sie ist sowohl durch die Bildung eines synzytiumähnlichen Filzwerkes um das Gewebe herum als auch durch die Auswanderung einer grossen Anzahl Herzmuskelzellen (Fig. 2) gekennzeichnet. In allen Präparaten wurde die Auswanderung von Zellen, welche nach genauer Untersuchung als Herzmuskelzellen identifiziert werden konnten, beobachtet. Der Umfang und die Dauer des Wachstums ist von der Festigkeit der Schichtdicke des Nährbodens abhängig. Während der zweiten Periode erfolgt eine langsame Vermehrung und Differenzierung der an ihrem neuen Sitz ansässig gewordenen Zellen. Man erkennt das Wachs-

[32]) Allerdings können durch eine besondere Vorbereitung der Gewebe und der Kulturen auch Ventrikelstücke von älteren Embryonen zur Pulsation gebracht werden. Die Ursachen, die einen Rhythmus hervorrufen oder unterdrücken, werden in einer späteren Arbeit besprochen werden.

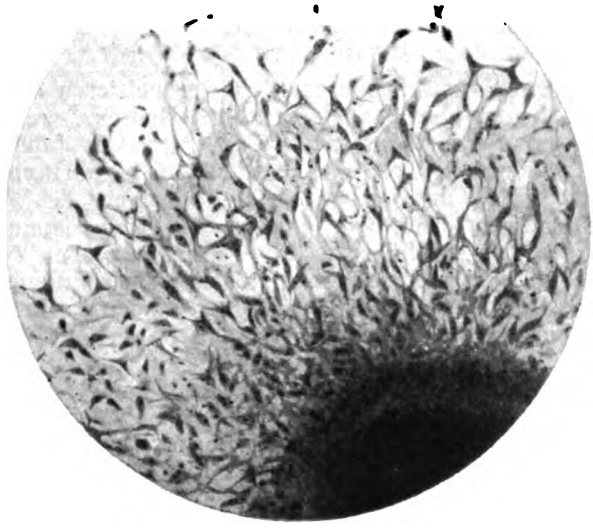

Fig. 2. Wachstum der Zellen aus einem Stück des Ventrikelmuskels eines 12 Tage alten Hühnerembryos. 8 Tage nach Herstellung des Präparates. In Hämatoxylin gefärbt.

tum an der Kernteilung und Protoplasmavermehrung der Zellen bzw. des Synzytiums.

Rhythmische Kontraktionen. In dieser Periode treten die rhythmischen Kontraktionen bei den ausgewanderten und differenzierten Zellen auf. Sie wurden in einem Fall schon am 5. Tag, in anderen Fällen erst am 14. Tage des Kulturlebens konstatiert. Die rhythmisch pulsierenden Zellen liegen stets im Fibringerüst und weisen ein durchsichtiges Protoplasma auf, welches vereinzelte Fetttropfen enthält. Unter 15 der nach der neuen Methode angefertigten Präparate, bei denen das Gewebe fortwährend mit frischem Serum umspült wurde, fanden sich rhythmische Kontraktionen neugebildeter Herzmuskelzellen bei 3 Präparaten, während nur bei 2 unter 150 der gewöhnlichen Hängetropfenpräparate dies der Fall war. Das häufigere Vorkommen funktionierender Zellen in den grösseren Kulturen hängt offenbar mit der bei diesen Kulturen länger andauernden Periode des lebhaften Wachstums zusammen. Es wurden nämlich Wachstumserscheinungen bei den grösseren Kulturen

bis auf den 30. Tag nach Herstellung des Präparates konstatiert, während sie bei den gewöhnlichen Hängetropfenkulturen zwischen dem 10. bis 18. Tage schon aufhörten. Wiederholte sukzessive Transplantation der Gewebekulturen verlängern zwar im ganzen die Dauer der funktionellen Tätigkeit, aber obwohl solche Transplantation jedesmal wieder mit einem neuen Auswuchs der Zellen begleitet ist, so ist doch ein solches Verfahren keineswegs imstande, weder das Leben der an Ort und Stelle neugebildeten individuellen Zellen, noch die bei diesen auftretenden Perioden funktioneller Tätigkeit zu verlängern.

Um auf die Bewegungserscheinungen, welche an isolierten Zellen beobachtet wurden, etwas näher einzugehen, sei einiges aus dem Protokoll der 12. Kultur angeführt. In diesem Präparat hatte eine einzelne Zelle, welche weit vom ursprünglichen Gewebestücke entfernt war, sich pulsierend bewegt, und zwar mit einem Rhythmus, der von dem des Synzytiums unabhängig war.

Die isolierte Zelle ist spindelförmig. Das eine Ende ist abgerundet, während das andere zwei ausgezogene Fortsätze aufweist, welch letzteren gröbere Fibrinfäden fest anhaften. Die Lage des einzigen Kerns wird durch eine leichte Ausbuchtung des Protoplasmas in der Nähe des runden Endes verraten. Das feinkörnige oder maschige Protoplasma weist einige perinukleäre, stark lichtbrechende Granula auf.

Die Phase der Kontraktion dauert beträchtlich länger als die der Erschlaffung. Die Erschlaffung erfolgt plötzlich, und erinnert an das Zurückschnellen eines gespannten Gummibandes. Bei der plötzlichen Entspannung der Zelle wirkt möglicherweise die Elastizität der Fibrinfäden mit. Die Zelle wird bei der Kontraktion um ca. $^1/_5$ ihrer Länge verkürzt, während ihr kurzer Durchmesser an allen Stellen scheinbar vergrössert wird. Das Intervall zwischen den zwei Phasen ist sehr klein. Bei mittlerer Vergrösserung sind die Bewegungserscheinungen im Mikroskop leichter zu verfolgen und man kann sie mittels Kinematographie aufnehmen, wie Braus angegeben hat.

Am 14. Tage hat das Synzytium als Ganzes gleichmässig pulsiert, während am 15. und 16. Tage nur noch zwei von einander getrennte Teile des Synzytiums pulsierten; zwar war die Frequenz des Rhythmus gleich geblieben, doch trat jetzt eine Phasenverschiebung ein, infolgedessen pulsierten die zwei Teile nicht mehr synchron. Daher ist anzunehmen, dass die zwischenliegenden Zellen nicht nur ihr spontanes Kon-

traktionsvermögen, sondern auch ihr Reizleitungsvermögen eingebüsst hatten.

Zusammenfassung: Die Herzmuskelzellen embryonaler Hühner können, nachdem sie Teilung und Differenzierung ausserhalb des Organismus erfahren haben, ihre spezifische Funktionstätigkeit sowohl als isolierte Zellen wie auch als zusammenhängende Zellmassen wieder aufnehmen. Der Rhythmus solcher Zellen stimmt mit dem des Herzens des lebenden Tieres überein. Die rhythmische Bewegung wurde nicht nur bei den ausgewanderten Herzmuskelzellen junger, sondern auch bei denen der 14 tägigen Embryonen beobachtet. Die Stücke selber, die aus dem Ventrikel der älteren Embryonen gewonnen sind, schlagen aber nicht, trotzdem die aus solchen Stücken isoliert ausgewanderten Zellen Kontraktionen ausführen.

Durch diese Untersuchung ist demnach ein direkter Beweis für die myogene Theorie des Herzschlages gebracht worden.

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THE EFFECTS OF ALKALOIDS ON THE DEVELOPMENT OF FISH (FUNDULUS) EGGS.

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THE goal of experimental embryology is the control of development, notwithstanding the fact that the majority of attempts in this direction have been failures. The embryo results from the interaction between the egg and its environment, and we might expect that a specific change in the medium would produce a specific change in the embryo. However, the organism is capable, to a great degree, of maintaining constant conditions within itself. Take, for example, the remarkable constancy in body temperature and composition of the blood of mammals.

One mechanism for the maintenance of constant chemical conditions within the organism is evidenced in the remarkable semipermeability of living cells. Overton found that volatile anæsthetics and free alkaloid bases, which are rarely encountered by cells, penetrate easily, whereas salts, with which cells are constantly in contact, do not ordinarily penetrate. I observed that neither salts nor anions penetrate the Fundulus egg, but that kations outside may be exchanged for those within.[1]

Herbst[2] thought he had found a specific effect of lithium salts on sea urchins' eggs in the production of exogastrulæ, *i. e.*, gastrulæ in which the archenteron is evaginated instead of invaginated. However, Driesch[3] produced the same results by a rise in temperature to 30° C. In this case the archenteron, or gut, sometimes shrank and disappeared, producing a condition known as anenteria.

[1] McClendon: this Journal, 1912, xxix, p. 295.

[2] Herbst: Zeitschrift für wissenschaftliche Zoologie, 1892, lv, p. 442, and Mitteilungen der zoologische Station zu Neapel, 1895, xi, p. 136.

[3] Driesch: *Ibid.*, 1895, xi, p. 221.

Gurwitsch [4] supposed that lithium salts produced a radially symmetrical gastrula in the frog's egg. Morgan [5] showed this not to be the correct interpretation, but the chief characteristic of these embryos is that the endodermal cells are not invaginated, and hence we might call them exogastrulæ.

In opposition to the above statements, Bataillon [6] denies that lithium or other salts or sugar act otherwise than osmotically, and states that isotonic solutions all have the same effect on frog's eggs.

Stockard [7] observed that lithium chloride causes an enlarged segmentation cavity, and retards the down-growth of the blastoderm over the yolk, in Fundulus embryos. He demonstrated that this is independent of the osmotic pressure of the medium, and in a later paper [8] stated that these abnormalities are "specific for the lithium ion in its action on this egg."

On the other hand, I produced "lithium embryos" with sodium chloride, calcium chloride, ether, acetone, and dextrose.[9]

Stockard produced cyclopic or one-eyed Fundulus embryos, and at first thought the abnormality due to the specific action of the magnesium ion,[10] but later obtained similar results by the use of volatile anæsthetics, and supposed them due to the specific action of anæsthetics.[11]

However, I obtained the same results, not only with several indifferent anæsthetics, but with sodium chloride, lithium chloride, and sodium hydrate, which are considered stimulating rather than anæsthetic in their action.[12] I found the order of effectiveness of kations (added to sea water) in producing cyclopia to be $Mg < Li < Na$. Since Hedin [13] found the same order in the rate of diffusion of these ions through dead ox gut, we may suppose their action in producing cyclopia probably to be physico-chemical. This may be true also of indifferent anæsthetics, since I showed that their effectiveness in producing

[4] Gurwitsch: Archiv für Entwicklungsmechanik, 1896, iii, p. 219.
[5] Morgan: *Ibid.*, 1903, xvi, p. 691.
[6] Bataillon: Archiv für Entwicklungsmechanik, 1901, xi, p. 149.
[7] Stockard: Journal of experimental zoölogy, 1906, iii, p. 399.
[8] Stockard: *Ibid.*, 1907, iv, p. 165.
[9] McClendon: this Journal, 1912, xxix, p. 297.
[10] Stockard: Journal of experimental zoölogy, 1909, vi, p. 285.
[11] Stockard: American journal of anatomy, 1910, x, p. 369.
[12] McClendon: this Journal, 1912, xxix, p. 289.
[13] Hedin: Archiv für Physiologie, 1899, lxxviii, p. 205.

cyclopia is proportional to their effectiveness in lowering the surface tension of water, and also proportional to their toxicity. The concentrations of salts and anæsthetics producing cyclopia are very near the lethal doses, although lower than those producing "lithium embryos."

Experiments.

The majority of drugs that have a specific action on the function of parts of the human body are included in the old group of alkaloids. Since the chemistry of many of these substances is unknown, the group cannot be well defined, and we will use the name as originally applied to substances of vegetable origin with basic properties. Many synthetic substitutes of the alkaloids might well be included in the group.

I have tried a number of alkaloids on the eggs of Fundulus heteroclitus, and produced the same abnormalities with each of them. Some experiments with glucosides were begun, but were cut short by the unusually early close of the breeding season. Comparative studies were made on the eggs of the sea urchin, Arbacia punctulata.

Of the alkaloids tried, caffeine and theobromine are xanthine bases, the remainder being derivatives of pyridine and quinoline. Stovaine (chlorhydrate of dimethylaminobenzoylpentanol), a synthetic product, was also used.

The alkaloids were usually made up in centi-molecular solutions in sea water, and various strengths obtained by dilution. The free base was used except in three cases. The hydrochloride of quinine was chosen. The sulphates of strychnine and morphine were used, and made up of half strength, since each molecule liberates two molecules of the free base. These salts of the alkaloids have the advantage of being more easily dissolved, and since sea water is alkaline, the free base is completely liberated in the solutions. Overton showed that alkaloids enter living cells only in the form of the free base, hence they are effective only in alkaline or neutral solutions.

In the stronger solutions the whole embryo degenerates, but in weaker solutions certain parts are affected more than others. Organs which arise early may degenerate, those which appear later may fail to develop. The circulatory system is the most affected, and may be suppressed to a greater or less extent, or may develop and not function, or may function for a while and then degenerate. The heart and

respiratory capillaries lie upon the surface of the yolk sac, and were more carefully observed than were the vessels which are obscured by the surrounding tissues of the embryo.

FIGURE 1. FIGURE 3. FIGURE 4.

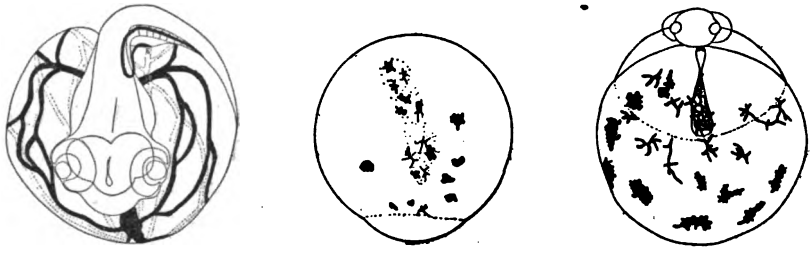

FIGURE 1. — Normal embryo of Fundulus heteroclitus, six days old. *S*, sub-intestinal vein; *D*, duct of Cuvier.

FIGURE 3. — Fundulus egg treated with $\frac{1}{80}$ molecular caffeine, showing the degenerating embryo one week old.

FIGURE 4. — Front view of embryo from $\frac{M}{160}$ caffeine, one week old. The heart beat, but the blood did not circulate.

A glance at the normal embryo will be useful for comparison. Fig. 1 shows the yolk sac circulation in a six-day embryo, the capillaries on the ventral side being indicated by dashed lines. The caudal vein passes on to the yolk sac as the sub-intestinal vein (*S*), and breaks

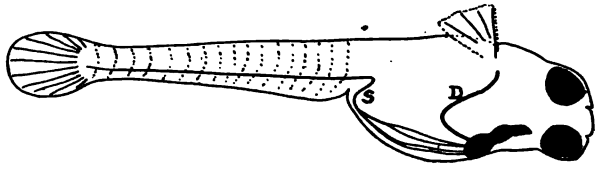

FIGURE 2. — The same embryo as Fig. 1, just hatched.

up into capillaries. The ducts of Cuvier pass out of the embryo and break up into capillaries, which anastomose with those from the sub-intestinal vein. This capillary plexus is reunited at the venous end of the heart, in front of the head of the embryo. It should be noted that in early stages the ducts of Cuvier draw their blood from the dorsal aorta through temporary connections.

As the embryo develops, the yolk is absorbed, and the capillaries on the yolk sac are gradually transformed into three large veins, the continuations of the ducts of Cuvier and the sub-intestinal vein. A transition stage is seen in an embryo just hatched (Fig. 2). The

right duct of Cuvier (*D*) is shown completed, but the sub-intestinal vein (*S*) is connected with the heart by four parallel veinlets, the remains of the capillary plexus. The anastomoses between the sub-intestinal vein and ducts of Cuvier have disappeared.

In the stronger solutions of alkaloids the embryos begin to develop normally, but sooner or later the cells begin to be loosened one from

FIGURE 5. FIGURE 6. FIGURE 7.

FIGURE 5. — Same embryo as Fig. 4, ten days old.
FIGURE 6. — Embryo from $\frac{M}{60}$ caffeine solution, eleven days old.
FIGURE 7. — Cyclopic monster from $\frac{M}{100}$ nicotine solution, four days old.

another, a condition called by Roux "framboisea." Such an embryo may live a long time, but gradually undergoes de-differentiation.

In Fig. 3, which represents such an egg a week old, the stippled area represents the embryo. The spots with blunt processes represent black chromatophores, and those with slender processes, red chromatophores. The blister on the yolk is the swollen pericardial cavity, and is the only means by which the head end of the embryo may be located.

Fig. 4 represents an embryo of the same age from a weaker solution, viewed from the front. The eyes are represented by the small circles. The semicircular areas, lateral to the eyes, are the distended ear vesicles, whereas the almost circular area reaching from the eyes to the dashed line across the middle of the yolk, represents the distended pericardial cavity. The elongate body, extending from the head ventralward, is the heart. It is beating, but no blood circulates, since no hollow vessels are connected with it. Erythrocytes, represented by stipple, lie in the arterial end of the heart. The lower or venous end of the heart is covered with red chromatophores.

In this same embryo three days later the eyes are degenerating, the left eye being represented by merely a thin smear of retinal pigment

(Fig. 5). The number of chromatophores on the ventral side of the pericardium and on the heart is greater than in a normal embryo. Owing to the swelling of the pericardial cavity, the heart is greatly elongated and its middle portion is transformed into a solid cord.

Sometimes but one eye completely degenerates, resulting in a condition which I have called secondary monophthalmia asymmetrica;

FIGURE 8.

FIGURE 9.

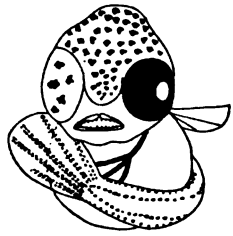

FIGURE 8. — The same embryo as Fig. 7, sixteen days old.
FIGURE 9. — Monstrum monophthalmicum asymmetricum, sixteen days old, from $\frac{M}{600}$ nicotine solution.

or the two eyes partially degenerate and then fuse together, secondary cyclopia.

Often the anterior region of the head disintegrates, and the cells wander away and become scattered over the pericardium. Such an embryo is shown in Fig. 6, and is atypical only in the fate of the eyes. The heart formed, but did not beat. The stippled area in the tail region represents a mass of erythrocytes. The pericardium was greatly distended and pressed against the ventral side of the head, to which it adhered. The anterior portion of the head disintegrated, and the eyes contracted into two spherical masses blackened by retinal pigment. By the eleventh day the eyes had fallen through the pericardial cavity and become grafted on to the heart.

Only one case of primary cyclopia occurred. This embryo, when four days old (Fig. 7), was apparently normal except for the single dumbbell-shaped eye on the ventral side of the head. The same embryo, sixteen days old, is shown from the front in Fig. 8. Although it had been removed from the nicotine solution when thirty-six hours old and had remained in pure sea water, frequently changed, for two weeks, defects began to appear in the circulatory system, and the circulation ceased entirely long before death. As shown in Fig. 8,

the pericardial cavity is distended and its contents turbid, and include a mass of erythrocytes around the venous end of the heart, represented by stipple.

The only case of primary monophthalmia obtained is shown at the age of sixteen days in Fig. 9. Except for the lack of the right eye, it is apparently normal. Even the right eye socket has developed in an apparently normal manner, but is covered by skin containing chromatophores.

TABLE I

Caffeine . .	50	80–200	Quinine . .	200	400
Atropine . .	90	100	Strychnine .	...	100–1600 (Saturated)
Brucine . . .	...	400 (Saturated)	.....	...	.....
Nicotine . .	350	400–700	Stovaine . .	800	1600

The alkaloids, even in very weak solutions, retard development. The toxic limits, and concentrations of solutions causing the abnormalities described above, are given in Table I. A saturated solution of theobromine produced no monsters, due to its very slight solubility, but, from comparative studies on the egg of the sea urchin, we may class it with caffeine.

To give a uniform basis for comparison, the data of only those experiments in which the embryos remained in the solutions thirty-six hours, beginning with the two-cell stage, are used. The "toxic limit" is the solution in which nearly all of the eggs are dead at the end of thirty-six hours. Each number in the table is the denominator of a fraction (of a molecular solution) whose numerator is 1. The middle column gives the toxic limits, and the last column gives the concentrations which suppress the circulation in various degrees, and cause the other abnormalities described above.

Comparative experiments were made on the eggs of the sea urchin, Arbacia punctulata. These eggs are more easily obtained, develop more rapidly, and are more sensitive to changes in the medium than are Fundulus eggs. All of the alkaloids, as well as stovaine and digitalin, produced the same abnormalities.

The strengths of the solutions are shown in Table II. The eggs were not removed from the solutions and returned to pure sea water, as in the case of the Fundulus eggs. The solutions of digitalin are

percentages, in case of the others the number represents the denominator of a fraction (of a molecular solution) whose numerator is 1.

In stronger solutions than those listed in the table, embryos died in segmentation stages. Even in those in the table development was enormously retarded, and the yolk granules dissolved more slowly, although ciliary activity did not appear to be reduced. Whereas

TABLE II.

Caffeine . .	500	800	Nicotine . .	3,200	6,400
Theobromine	.....	Saturated	Quinine . .	50,000	55,000
Atropine . . .	3,200	25,600	Strychnine . .	25,600	51,200
Brucine . . .	12,800	51,200	.....	.....	.....
Cocaine . . .	6,400	25,600	Stovaine . .	12,800	102,400
Morphine . .	800	6,400	Digitalin . .	.0004%	.0001%

normal embryos, if not fed, starve to death in a few days, some embryos in the alkaloid solutions live several weeks. After the first few days a distention of the body cavity commences and may continue until the ectoderm becomes a very thin-walled vesicle.

In the solutions listed in the middle column plutei were not produced, although rudiments of the skeleton, in the form of tri-radiate stars, sometimes appeared. Some exogastrulæ were produced. In case normal invagination of the endoderm did take place, the gut was never normally differentiated, but usually degenerated into a solid mass of cells.

In the solutions listed in the last column plutei were formed, but there was an early disarrangement of the mesoderm cells, so that the resulting skeleton was abnormal. Since there was an enormous number of forms of the skeleton, they cannot be described here.

Conclusions.

It has been shown above that the very different organic compounds used, belonging to both the aliphatic and the carbocyclic series, although included in the old class of alkaloids, have the same morphological effects on the eggs of Fundulus heteroclitus. Loeb has obtained the same abnormalities in solutions of potassium cyanide and has reared similar monsters from eggs fertilized with foreign sperm.

It might be supposed that these effects follow any injury to the Fundulus egg. The distention of the pericardium follows the application of various salts. I have produced it in frog's embryos by mechanical injury. The distention of other serous cavities sometimes follows the application of certain salts and anæsthetics. Loeb prevented the heart beat with potassium salts. Some embryos in ammonium salts were observed by Stockard never to develop a heart beat, and in some in magnesium salts the circulatory system degenerated.

It may be possible that the same abnormalities can be produced by any chemical treatment. However, the quantitative data show striking differences in the effects of different substances. Using solutions of equal toxicity, almost 100 per cent of embryos in ethyl alcohol may show primary defects in the eyes, whereas such defects are seen in not more than one tenth of 1 per cent of embryos treated with alkaloids. On the other hand, the abnormalities described in the circulation may occur in nearly 100 per cent of embryos treated with alkaloids.

Thousands of eggs of Fundulus heteroclitus were placed in solutions of each of the alkaloids enumerated, and the eyes of each embryo were carefully examined, but only one case of primary cyclopia and one of primary monophthalmia asymmetrica were observed. These two occurred in the same batch of eggs treated with nicotine. I subsequently used this same concentration of nicotine on the eggs of many different females without reproducing these abnormalities.

Stockard obtained very different percentages of cyclopia in eggs treated with magnesium salts during different seasons or parts of seasons. He once thought this due to accidental variation in the concentration of the solutions. This could not have been the case at least in my experiments, as I used a finely graduated series of concentrations extending a great distance on each side of the apparent optimum for producing cyclopia. In numerous experiments covering an entire season, I failed to obtain as high a per cent of cyclopia with magnesium chloride as recorded by Stockard. By careful measurements of specific gravity, I found that the varying results were not due to differences in density of the sea water. The water I had been using was obtained directly from the sea in glass vessels, but as a control I used sea water drawn from the same pipe as that used by Stockard. This was repeated many times, and it was demonstrated that heavy metals in the water did not account for the differences.

The cause of the varying results must be that different batches of eggs have not the same tendency toward cyclopia, just as the individual eggs laid by the same female at the same time vary in this respect. Perhaps some Fundulus eggs would produce cyclopic embryos without any laboratory treatment, as I have found many cyclopic smelt embryos in the natural breeding places. It would not be safe, therefore, to draw conclusions from two individuals in many thousands. Attention should be directed rather to the quantitative data.

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ECHINOCHROME, A RED SUBSTANCE IN SEA URCHINS.

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INTRODUCTION.

My interest in echinochrome arose from studies in permeability. In the same way that haemolytic agents cause haemoglobin to leave the red blood corpuscles, so do cytolytic agents cause echinochrome to leave the cells containing it. R. Lillie is of the opinion that this is due to the action of the cytolytic agent in increasing the permeability of the cell surface.

In the elaeocytes, wandering cells of the body fluid of *Arbacia punctulata*, the cytoplasm is crowded with spherical chromatophores. Some of these may be colorless, but usually they are colored bright red with echinochrome. Similar chromatophores, though not so close together, occur in the eggs. In the unfertilized egg they are evenly distributed throughout the cytoplasm. But after fertilization, the chromatophores all migrate to the surface within half an hour. During cleavage of the egg, they are massed in the cleavage furrows. The pigment occurs also in the test of this sea urchin, and gives the animal the characteristic color, which varies from a bright red (especially in young individuals) to a dark red, and may be almost black in old specimens.

In reference to the fact that the pigment may be caused to leave the chromatophores and pass into the cytoplasm and thence into the medium, the following questions may be asked: (1) How is the pigment held in the chromatophores? (2) What is its function? (3) What is its chemical nature? The present paper is concerned with these questions.

HISTORICAL.

Echinochrome was studied spectroscopically by McMunn,[1] who found it in the elaeocytes of the sea urchins, *Strongylocentrotus lividus*, *Amphidotus cordatus*, *Echinus esculentus?* and *E. sphaera*. The spectrum showed faint absorption bands, which varied with different solvents and different reactions of the same solvent. McMunn thought that he noticed changes in the spectrum on the addition of powerful reducing agents, such as stannous chloride, and concluded that echinochrome functioned as an oxygen carrier. However, the absorption bands in its spectrum are difficult to make out except in absolute alcohol (or glycerine) and in this solvent I observed that stannous chloride caused a precipitation of the pigment, which interfered with the examination.

A. B. Griffiths[2] attempted an elementary analysis of the substance. He dried the elaeocytes and extracted them with chloroform, benzol or carbon bisulphide. On evaporation of the solvent he analyzed the substance without further purification, although evidently it contained many impurities. From four analyses, he deduced the formula $C_{102}H_{92}N_{12}FeS_2O_{12}$, which would make C = 67.8 per cent, H = 5.5 per cent, and N = 9.3 per cent. He states that on boiling with mineral acids it is transformed into haematoporphyrin, haemochromogen and sulphuric acid (E + acid = $2C_{34}H_{34}N_4O_6 + C_{34}H_{37}NFeO_6 + H_2SO_4$). Griffiths agrees with McMunn that echinochrome is an oxygen carrier, and states that the oxygen is held rather firmly, and in nature is removed only by the reducing action of the cell containing the pigment.

EXPERIMENTAL.

The pigment in the elaeocytes, eggs and tests of Arbacia, shows no absorption bands, but after extraction it shows very similar bands in its spectrum to those described for echinochrome by McMunn. He published drawings of the spectra and measured the wave lengths corresponding to the *edges* of the bands. It is well known that bands become broader as the solution is more concentrated, and for that reason I measured the wave length of a line of the spectrum corresponding as nearly as could be determined to the *center* of each band. By taking the mean between the wave lengths of the edges of the band in McMunn's data I have compared his with mine. The discrepancies may be accounted for,

[1] McMunn: *Quart. Journ. Micro. Sci.* (2), xxv, p. 469, 1885; xxx, p. 51, 1889.

[2] Griffiths: *Compt. rend. soc. biol.*, cxv, p. 419, 1892; *Proc. Roy. Soc. Edinburg*, xix, p. 117, 1892; *Physiology of the Invertebrata*, New York, 1892; *Respiratory Proteids*, London, 1897.

first by the fact that the mean is not the exact center of the band in a prism spectrum, and secondly there is a personal equation in observation. I found the pigment extracted from elaeocytes, eggs or tests to give about the same spectra, though a few isolated observations seemed to vary. These might have been due to decomposition products with different spectra.

	ETHER		ABSOLUTE ALCOHOL								H_2O			
			Neutral			+HCl		$+NH_3$			+HCl		$+NH_3$	
My data...	5296	4844	5504	5302	4844	5296	4844	5154	4844		5296	4844	5154	4844
McMunn ..			5512	5128	4848	5370	4998	5205	4848					

Neither McMunn nor Griffiths succeeded in crystallizing echinochrome. Dr. A. P. Mathews had observed that on the addition of iodine in potassium iodide (KI_3) crystals form easily. In 1910 I obtained quantities of these crystals, but did not succeed in recrystallizing them without great loss by the formation of amorphous masses. The iodine compound in absolute alcohol showed an additional, but very dim band in the spectrum (wave length 5628 or 5696). It crystallized in red or orange needle-like crystals, triangular in cross section, sometimes rhombic in side view and often forming rosettes. They were but slightly soluble in water unless hot or containing acid, soluble in absolute alcohol (the rhombic crystals seeming more soluble than the needles) and slightly soluble in ether. If a solution in water is shaken with ether the latter is not colored. If an alkali is added to the KI_3 solution no crystals are formed (due to combination of the base with the echinochrome) but HCl does not prevent their formation.

Some of this iodine compound which was kept for several months in a dry state became more soluble in ether and crystallized in flat thin, red or orange rhombic plates. Perhaps the substance had decomposed with the liberation of iodine, for I succeeded in crystallizing the mother substance and obtained the same plates, in addition to red or orange needles, never triangular in cross section, but sometimes forming rosettes.

I extracted echinochrome from the tests with strong, slightly acidulated alcohol and purified it by repeated precipitation with

alkali and solution in acid alcohol, and filtration.[3] Finally I dissolved the precipitate in water plus HCl, filtered and shook the solution with ether. The ether did not remove all of the echinochrome and the formation of haptogen membranes caused much loss of material. The ether was evaporated at room temperature, as heat seemed to decompose the substance. Occasionally a few crystals formed at the edges of the solution but the main mass of the residue was amorphous.

The next season (1911) I tried to purify echinochrome without the use of acids or alkalies. The body fluid of the sea urchins was allowed to clot and the elaeocytes thus obtained were placed directly into acetone, which extracted the pigment. The extract was filtered and evaporated at room temperature. The residue was washed with carbon tetrachloride (which does not easily dissolve echinochrome) to remove fats, and again dissolved in the smallest quantity of acetone and filtered to free it from traces of lecithin. This solution was evaporated, dissolved in absolute ether and filtered to remove salts, evaporated to constant weight and analyzed by Dennstedt's method. A mean of two analyses gave: C = 51 per cent, H = 7.7 per cent. The echinochrome purified by precipitating with alkali gave C = 53.3 per cent, H = 4.4 per cent, N = 1.5 per cent. The nitrogen was determined by Kjeldahl's method and therefore may not be reliable, since the constitution of the molecule is unknown. Traces of sulphur and phosphorus, possibly due to impurities were found, but no iron. The ether-soluble crystals from spontaneous decomposition of the iodine compound gave C = 57.9 per cent, H = 6.5 per cent.

It was stated above that echinochrome is precipitated by alkalies in alcohol. I precipitated echinochrome with NaOH in 95 per cent alcohol and washed in the same alcohol to remove the excess of NaOH. From the amount of NaOH that was neutralized by the pigment I concluded that it combined with from 18 to 25 per cent of Na. Analysis gave C = 31.5 per cent, H = 6 per cent, Na = 19.5 per cent. Therefore we may say that the echinochrome behaves as an acid, or else is amphoteric. The former view is

[3] Alkali does not precipitate it in water; the particular base was immaterial, ammonia was added but the presence of sea salts allowed the liberation of other bases.

supported by the fact that on passing an electric current through the aqueous (colloidal) solution, the echinochrome shows a negative charge (is anodic) and again, if histological sections are placed in such a solution the acidophile portions are stained more strongly than the remainder. In fact its behavior is very similar to that of a weak solution of eosin, except that it is very easily washed out by alcohol.

However the substance is probably amphoteric (the acid character being stronger than the basic) since its aqueous solution is precipitated by phosphomolybdic and phosphotungstic acids but not by tannin.

From the analyses given above it would seem that no one has succeeded in obtaining echinochrome in a reasonably pure state. It is very unstable and probably breaks up into a host of decomposition products all having practically the same spectrum. If it is kept in the dry state for a great length of time, or is evaporated on a bath not over 50° for a shorter time, part of it becomes insoluble in ether but not in alcohol.

When heated in the combustion tube it first melts, then boils and sublimes as crystals on the top of the tube, then very soon turns brown and chars. After being crystallized from a solution in ether the crystals often become smaller and irregular in outline. Perhaps the crystals evaporate or lose water of crystallization, but I think that both these possibilities are improbable. The crystals may decompose into an amorphous substance.

On first obtaining crystals, I feared that they were crystals of some other substance merely colored by echinochrome, but this seems impossible from later observations.

In extractions made for the purpose of studying the lipoids in Arbacia eggs, red or brown substances (echinochrome or its decomposition products) appear in every fraction, rendering analysis difficult and indicating the instability and wide solubility of the substance.

In order to test the statement that it is an oxygen carrier I separated the cells from 50 cc. of body fluid by the centrifuge, and mixed them with sea water to make 50 cc. This suspension, and 50 cc. of sea water as a control, were placed in two similar graduated tubes. The air was pumped out for six hours (until the water boiled), air was then admitted and the tubes sealed. They

were shaked one-half hour and the volume of air measured at atmospheric pressure. The suspension had lost 1.25 cc., the control only 0.8 cc. In another experiment the suspension lost 0.95 cc. and the sea water 0.8 cc. It was thought that in the absence of oxygen the cells would take the oxygen from the echinochrome. However no color change could be observed with the naked eye or the spectroscope, and the greater absorption of air by the suspension may have been entirely due to oxidation in the cells. In similar experiments, with an aqueous solution of the pigment, and distilled water for a control, and using pure oxygen, the two tubes gave the same absorption, as shown by two examples:

Oxygen absorbed by H_2O	1.0
	1.1
Oxygen absorbed by echinochrome	1.0
	1.15

The question, how echinochrome is held in the chromatophores, cannot be fully answered. The chromatophores when free from pigment are highly refractive and stain strongly with the intravitam stain, neutral red, and when fixed they stain strongly with Delafield's haematoxylin, indicating a lipoid nature. The pigment may be in solution in the lipoid.

The fact that the spectrum is different (shows no bands) in life from the spectrum of the extract may indicate chemical combination of the pigment with the chromatophores. The fact that echinochrome stains acidophile tissue may show a possible mode of such combination, if it be found that the chromatophores contain bases. However I do not think we can rely on the spectroscopic evidence, for the absorption bands are very faint in aqueous solution unless it be alkaline, and the cells containing the pigment interfere with the passage of light and make the observation difficult. I have never seen absorption bands in echinochrome extracted from the fresh cells with distilled water. The same statement is made by McMunn. If the substance is held by chemical combination why does it come out so easily?

The same argument may be made against the possibility that the echinochrome is held in the chromatophores because it is more soluble in them than in water. When the cell is stimulated mechanically or chemically the pigment comes out of the chromato-

phores with explosive rapidity. The cell need not be killed to accomplish this. The mere act of normal fertilization causes some of the chromatophores in the egg to lose their pigment.

The only alternative hypothesis I know of is, that the pigment is manufactured in the chromatophore, and cannot normally get out because the surface of this body is impermeable to it. An increase in permeability of the chromatophore allows the pigment to escape. Such an increase in permeability might be due to an aggregation change in the colloids of the limiting membrane or surface film.

Echinochrome is held in the chromatophores of the sea urchin's cells probably in the same way that chlorophyll is held in the chromatophores of the green plant cell.

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PREPARATION OF MATERIAL FOR HISTOLOGY AND EMBRYOLOGY, WITH AN APPENDIX ON THE ARTERIES AND VEINS IN A THIRTY MILLIMETER PIG EMBRYO

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THREE FIGURES

The essential of a good course in histology or embryology is good material. Fresh human material should never be allowed to go to waste, but it may be at times very inconvenient to put it up in a variety of fancy fixing fluids.

Perhaps the best general cytoplasmic fixer is formalin of 10 to 20 per cent (4 to 8 per cent formaldehyde). If material so fixed is not soaked too long in alcohol of high concentration, it may be used as fresh tissue in special technique to show fats or mitochondria. In fact the formaldehyde alone makes unsaturated fats and lipoids less soluble in clearing fluids. On the other hand, if the washing in water is omitted, the structure of resting nuclei is well enough preserved for ordinary purposes.

Commercial formalin contains formic acid, which, although developing a more beautiful nuclear structure, may begin to cytolyse the more delicate cells before they are sufficiently fixed by the formaldehyde. This is especially noticeable in erythrocytes—haemolysis, or escape of haemoglobin, occurring in parts of the tissue. Furthermore, acids swell fresh white fibrous tissue. It seems worth while, therefore, to neutralize the formol, and this may easily be done by adding slack lime ($CaCO_3$) or magnesia, and filtering.

Doctor Ferguson first called my attention to the fact that kidney swells in many fixing fluids, whereas it is commonly supposed

that the majority of tissues shrink a little. Death of isolated cells as seen under the microscope may be accompanied by swelling (cytolysis) or contraction. In every case, an increase in permeability to some substances occurs, but I found that during the early stages of cytolysis of the sea urchin's egg, it remains very impermeable to salts. Dead animal or plant membranes are more permeable to water than to dissolved substances, but apparently some living cells are impermeable to water. The Fundulus egg, if transferred from sea water to distilled water, does not burst, though it is certainly not capable of resisting the enormous osmotic pressure of its internal salts. Since I found this egg to be impermeable to salts, it must also be impermeable to water (it is permeable to kations, but for every kation that comes out, the electrical equivalent must go in). If such a cell became, on death, permeable to water, the osmotic pressure of its internal dissolved substances might cause it to swell. If a Paramoecium be killed by an ordinary fixing fluid, even though it be hypertonic, the protoplasm first coagulates, then the whole animal swells a little. This may be what happens to some tissue cells, and I found that it is not always prevented by the addition of 0.9 per cent sodium chloride to the fixing fluid. Therefore I supposed the swelling due to the osmotic pressure of some contained substance of large molecule, and experimented with the addition of cane sugar to neutral formol. By this means the cytolysis of adult convoluted nephric tubule cells is prevented, and the general fixation is good except that some nuclei may be slightly shrunken. This fluid may be used for all adult tissues and embryos, and is easily prepared as follows:

Formol	100–200 cc.
Cane sugar	20–40 grams
Slack lime ($CaCO_3$) or magnesia	about 1 gram
Water to make 1 liter.	

If the shrinkage of a few nuclei is very objectionable use only 20 grams of sugar. This fluid has the advantage that tissues and embryos float in it and therefore do not become distorted.

If the whole kidney of a fetus be fixed in the above mixture or any other fixing fluid, the cells of the convoluted tubules will

swell until they fill the lumen. This brings us to a well known point that is often neglected. Tissues should be cut into as thin slices or pieces as is practicable and the cells not injured in the cutting. Fetal tissues are especially delicate. They should be cut with a very sharp thin blade and lifted on the blade into the fixing fluid.

Many workers object to formalin because it "causes" a homogeneous appearance to protoplasm. The ultra microscope has shown that, aside from evident granules, living protoplasm is homogeneous, contrary to Bütschli and others. There are persons who now accept formalin for cytoplasmic fixation but say that it "does not fix nuclei well." Some structures may be seen in living nuclei. I have studied many nuclei with high powers and with the ultra microscope, yet I cannot decide what form of fixation corresponds most closely to the living structure. Both cytoplasm and nucleus of a living erythrocyte of a frog is homogeneous when examined in serum or uncoagulated plasma with the ultra microscope. Sooner or later bright points or clouds appear on or in the nucleus, but this is usually associated with change of nuclear form and is evidently due to injury.

Formaldehyde not only does not coagulate protoplasm but renders it more difficult to coagulate. It also makes lipoids less soluble in clearing fluids. However, I find an after-treatment with Müller's fluid or some other oxidising fluid necessary for the preservation of lipoids, the amount of oxidation necessary depending on whether mitochondria, myelin or fats are studied.

Ordinary staining depends on the fact that all protoplasm treated with acid, stains with acid dyes, whereas certain parts take also basic dyes. Many staining solutions contain free acid, but tissues stain more quickly if they are previously treated with acid. For this reason we put everything into the formol mixture and after a few hours transfer part of it to Bouin's fluid: This tissue is finally stained on the slide in haematin and eosin. The alum haematin lake is usually so strong that it stains in three minutes, but the eosin is so much diluted that twelve hours are required to stain and in this time smooth muscle stains less intensely than white fibrous tissue The acid in

Bouin's fluid causes the tissue to stain more brilliantly but if the fresh tissue is put into Bouin's fluid the blood in some of the vessels will be laked. Part of the material is transferred from the formol mixture to Müller's fluid and subsequently stained with iron hematoxylin to show the lipoids (mitochondria, etc.).

Ordinarily, the student is shown two dimensions of a piece of tissue or embryo, and left to imagine the third. Though whole mounts of chick embryos are handed out, cleared pig embryos, and blocks or thick sections of certain tissues are even more useful. For a solid mount, the object should be placed in a dish of balsam or damar dissolved in benzol and protected from dust until it evaporates down to sirupy consistency, then mounted in the usual way. By this means the necessity of rings or other supports to the cover glass is avoided, and drying out or great shrinkage prevented.

All of the solid mounts turn yellow with age, but a number of highly refractive fluids may be obtained that are colorless. These are listed, with their refractive indices, in Landolt-Bornstein; Behren's Tabellen; and Lee's Vade Mecum. The higher the refractive index the better, for if in any case a lower index is desired, this may be obtained by the addition of paraffin oil or xylol (or water in case of aqueous media). It may be noted here that, whereas the process of clearing in a mixture of oil of wintergreen (Gaulteria) and benzyl benzoate has been patented in Germany and is widely known under the name of the patentee, wintergreen was first used by Stieda in 1866, and the synthetic oil (methyl salicylate) recommended by Guéguen in 1898, and is noted in various books on technique.

Methyl salicylate is permanently colorless, and comparatively inexpensive, and ideal for a fluid mount. If rings are cemented on slides with shellac or liquid glue and allowed to dry, they are not loosened by the oil. Paper rings soaked in shellac or glue will do, but rings may be cut from lead pipe with an ordinary saw or a bone saw if the proper size of glass rings are not at hand. The shellac must be dry before adding the oil, which must be free from alcohol. I prefer glue.

If the tissue is hardened in alcohol, thick sections may be cut free-hand. Thick sections are often better unstained, especially

if injected, and much detail may be made out by partly closing the diaphragm of the microscope. If stained with very dilute haematin containing much acid, connective tissue is colorless and cytoplasm nearly so, whereas nuclei may be readily distinguished. In this way blood vessels and glands in areolar tissue are caused to stand out sharply.

Whole mounts and thick slices are especially useful in embryology, and are a necessity unless one is contented with teaching the third dimension with models. The larger the embryo, the more attention must be paid to the clearing medium in order to distinguish internal structures. Methyl salicylate is admirable for pig embryos of all sizes and even for small fetuses. I found ethyl salicylate to be as good if not better, but it is more expensive. Canada balsam has about the same refractive index ($^{n}D = 1.535$) as methyl salicylate ($^{n}D = 1.536$), but darkens with age.

Embryos may be placed directly from absolute alcohol, benzol, xylol, toluol or chloroform into methyl salicylate, but in order to obtain the proper refractive index, the preliminary fluid must all be removed. This may be evaporated, or washed out with more wintergreen. Benzol is to be recommended because it is cheapest and evaporates out most easily. The evaporation may be hastened by an air pump, which also removes any air bubbles that may get into the specimen. These bubbles expand and are absorbed after the pump is disconnected, or by successive pumpings. An ordinary air pump will cause the benzol and air to boil out. A water-suction air pump (aspirator) will suffice but a float valve and safety bottle should be interposed between the pump and the specimen to prevent the back flow of water. An exhaustible desiccator is convenient for holding large embryos while they are being pumped out. If the cover is well ground, the oil will seal it sufficiently, and vaseline should not be used.

Most of the internal organs may be distinguished in unstained embryos by cutting down the light. The individual cells of mesenchyme, cartilage and blood may be seen; the cellular structure of the neural tube is indicated by radial striations and the

larger nerves appear as bundles of fibers. Some organs in smaller embryos are made more distinct by staining with very dilute alum haematin containing a large amount of acid.

Even in quite small embryos, many of the blood vessels may be traced by the blood cells, and the large empty veins followed as cavities. However, with the smaller vessels this becomes more laborious than serial sections. On the other hand, the injection of small embryos for class use means quite an outlay of time. Therefore, it seemed necessary to find some way to fix the haemoglobin, and keep the vessels full, in order to distinguish the vessels by the color of the blood. I found that *the same method that prevents the cytolysis of nephric tubule cells prevents haemolysis.*

Living embryos are obtained, the amnion opened, the placenta squeezed to force the blood into the embryo, and the umbilicus tied or clamped. Artery clamps are too strong and pinch off the cord. (I made clamps out of wire (lower part of fig. 1) in order to avoid tying so many cords at the slaughter house. The clamp may be removed in half an hour and used again.) The embryo is dropped into the neutral-formol-sugar mixture described above, and left until thoroughly fixed. In case of a fetus, part of the skin should be torn off after the superficial blood vessels are fixed, to insure penetration of the formaldehyde. A hole may be made in the skull by slicing off a small piece tangentially or by a sagittal cut near (to the right of) the median plane. Large fetuses, unless skinned completely, will have to be scraped to remove the pigment layer.

Transfer the specimen after washing, or directly, from the fixing fluid to alcohol of about 70 per cent. After they have hardened in 80 or 95 per cent alcohol it is well to split the large specimens by a sagittal cut a little to the right of the median plane with a very thin bladed knife. The dehydration with higher alcohols should be slow enough to prevent shriveling.

By this method the blood retains its color, and although it does not take the place of injection, it is a great help to the student. I have inserted three figures to show what can be seen in such specimens.

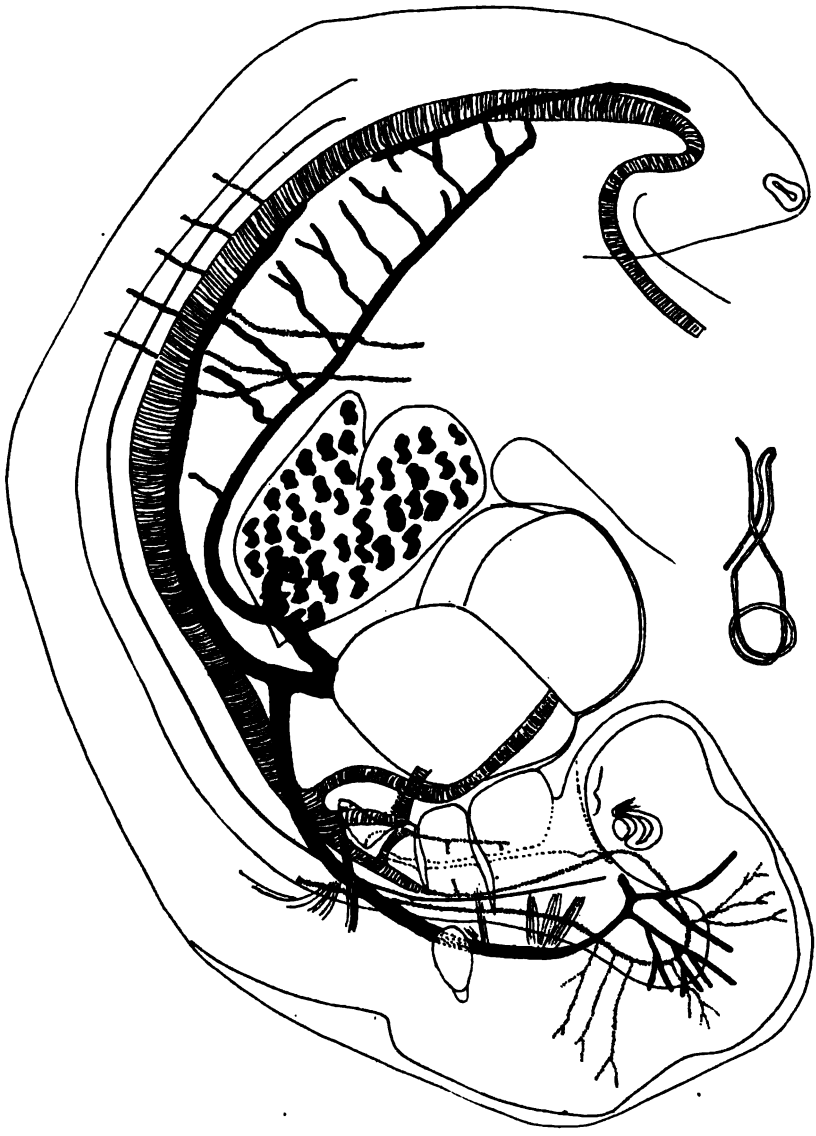

Fig. 1 Left half of a pig embryo 7 mm. long after clearing. The veins are black, the arteries cross-striated, the 5th, 7th, 8th, 10th and 11th cranial nerves are longitudinally striated, the notochord is represented by a heavy line and the fore gut by a dashed line. The sinuous line ventral to the embryo represents a wire (partly open) clamp used in clamping the umbilicus.

Figure 1 represents a pig embryo about 8 mm. long. The nerve tube, fore gut, mesonephros, liver, heart, eye and ear are clearly seen. The arterial system and part of the cardinals and subcardinals can be distinguished. The notochord is distinct, and the 5th, 7th, 8th, 10th, and 11th cranial nerve roots can be made out. Figures 2 and 3 are described in the appendix.

I have prepared hundreds of pig embryos and fetuses in this way, and also injected many with india ink and cleared them in wintergreen oil. A completely injected fetus can only be studied in comparatively thin (freehand) sections. Various degrees of partial injection are very useful to show the larger vessels, but these may be seen in the uninjected fetuses. The left side of an uninjected fetus which has been cleaved a little to the right of the median plane, will show the general circulation, except in the liver. The larger vessels in the liver may be seen by removing the lateral portions and passing a strong light through the remainder (an arc light is excellent), or the liver may be removed and cut into slices. In injected specimens the liver is hopeless.

I washed with alcohol the blood out of the vessels of a fetus 4 inches long and cleared it in wintergeen oil, then injected it with mercury. This method has the advantage that the extent of the injection may be watched and controlled.

The injection may be limited by using a coarse granular pigment that will not go into the capillaries. A gelatine mass is not absolutely necessary to hold the pigment. A light colored opaque pigment has the advantage that it may be seen by transmitted or reflected light.

The arteries may be injected and the haemoglobin fixed in the veins, giving handsome specimens. If it is desired to show only the injection, no formalin should be used. Much of the haemoglobin may be dissolved out by putting the fresh specimen into weak alcohol or alcohol and acetic acid. All of the haemoglobin may be removed with dilute acetic acid provided an injection is used that is not affected by this acid.

APPENDIX

ON THE ARTERIES AND VEINS IN A 30 MM. PIG EMBRYO

The method of fixing the haemoglobin and clearing in wintergreen oil to show the course of the vessels has been especially successful in case of pig embryos of about 30 mm. length Figures 2 and 3 show the larger vessels of the median plane and left side of one of them. The courses of most of the vessels approach the type of the adult pig and show distinctions in topography from those in man. The common carotid artery and (right) innominate artery arise from a common trunk, the brachiocephalic artery. The posterior inferior cerebellar artery arises from the basilar instead of from the vertebral.

Notwithstanding the great development of the vena cava, the left posterior cardinal is of considerable size. The right cardinal (not figured) is smaller. The thoraco-epigastric vein is divided into two parts, one of which drains anteriorly into the internal mammary.

The vessels of the limbs could not be completely followed, but enough was seen to demonstrate that they differ very much from those in the adult.

Besides the vessels, the mouth cavity, brain, eye, endolymphatic labyrinth, lungs, mesonephros, kidney, testis and penis are outlined in the figures.

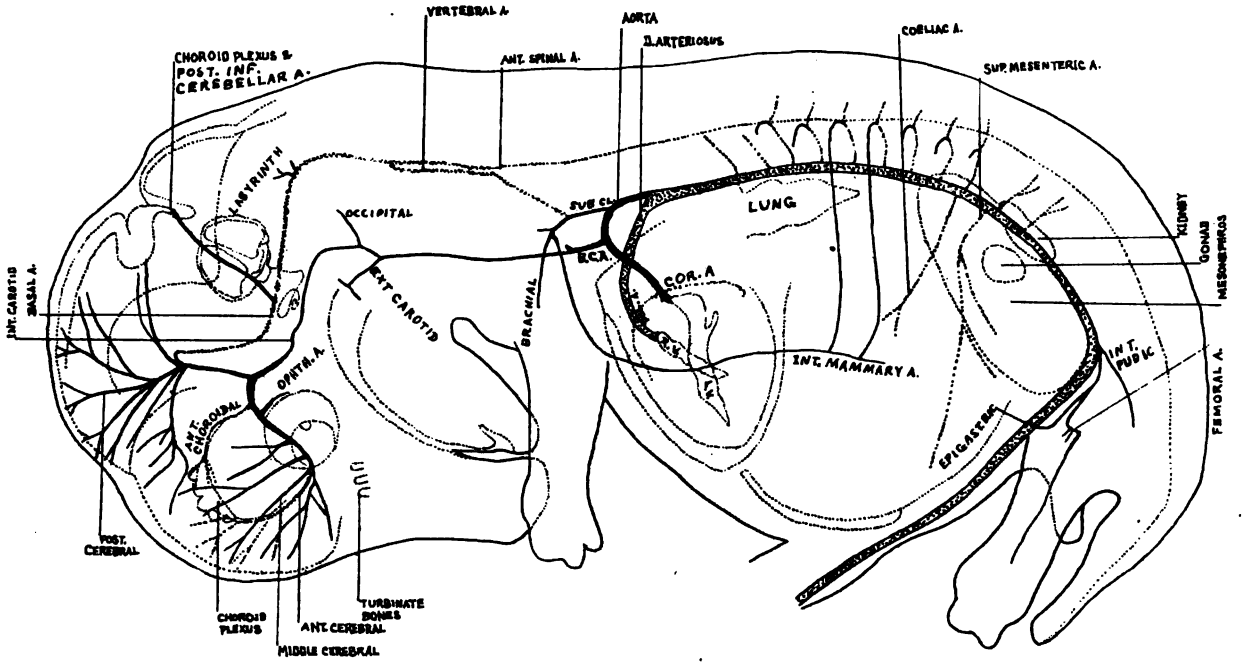

Fig. 2 The arteries of median plane and left side of a pig embryo 27 mm. long after clearing. The arteries farthest from the observer are stippled. The musculo-fibrous coats are not represented, and some of the arteries are so contracted as to almost obliterate the lumen. *B.C.A.*, brachio-cephalic artery; *COR.A.*, coronary; *SUBCL.*, subclavian; *PUL.A.*, pulmonary; *OPHTH.A.*, ophthalmic; *LV.*,*RV.*, cavities of left and right ventricles.

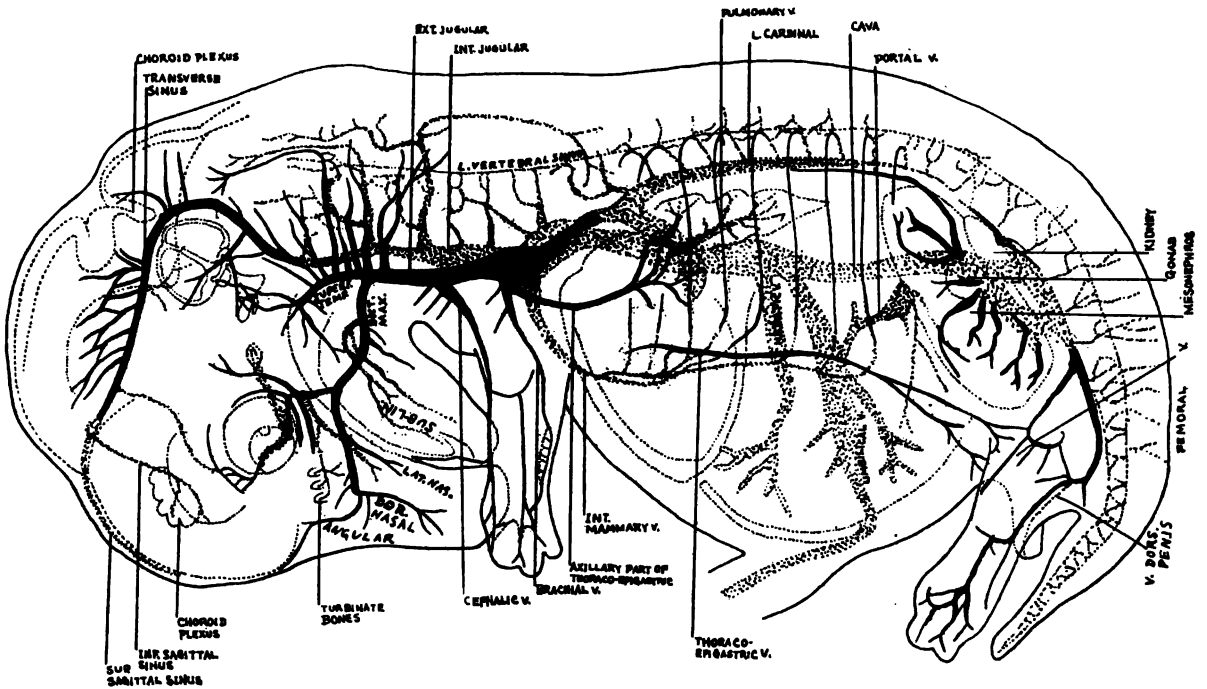

Fig. 3 Veins of median plane and left side of a pig embryo 27 mm. long after clearing. Each vein is represented by its lumen only, but some have been purposely made smaller for clearness in the figure. The representation of the veins between the mesonephroi is somewhat diagrammatic.